The effects of targeted therapy on cell viability and apoptosis on CML and AML cell lines

Thesis submitted in accordance with the requirements of the University of Chester for the degree of Doctor of Philosophy by

Paolo Marsico

January 2018

DECLARATION

The material being presented for examination is my own work and has not been submitted for an award of this or another HEI except in minor particulars which are explicitly noted in the body of the thesis. Where research pertaining to the thesis was undertaken collaboratively, the nature and extent of my individual contribution has been made explicit.

Signed:

Date:

ACKNOWLEDGMENTS

I would like to thank Professor John Williams and Dr. Elyse Ireland for giving me the opportunity to carry out this work; their supervision, expertise and in, most cases, their infinite patience have led me to finish this work. For this, I will be always grateful.

I would like to thank all the staff of Thomas Building and of the Medical School for the technical support, with a special mention to Emily. I would also like to thank all the "Lab Rats" with whom I shared laughs and tears in the lab: Bura, Muneera, Gareth, Neha, Emmanuel, Sheah, Monsurat and Hanady. A special thanks to Michelle and Taha for their support and guidance and to Prince for being my special lab buddy.

Obviously, a profound thank you goes to my Mum for her sweetness, never ending support and for being just my mum. Thanks to my Dad for being the man I always look at as a model in my life; if I will ever be even close to the man he is, I will be incredibly lucky. A special thank goes to my twin brother Matteo; there are no words to describe our relationship, which goes beyond imagination. Thanks for being not only a brother, but a friend, in every single moment of my life. This work happened also because of you. Another special mention to Rosa, for being part of our family and for being an inspirational figure in my life.

I would also like to thank my sweet Aunt Margherita, Giacomo and Carlo especially for his technical support and for his invaluable patience. Also, I would like to thank my uncle Flavio and Grandad Carlo, who are a constant inspiration for me and to Silvia; particularly, I would like to thank my cousin Alessandra for being the sweetest girl I know and for keeping me occupied with "experiments" even when I am home. A thank you goes to my uncle Mauro, Monica, Andrea, Miriana and Manuela who are always in my heart although I am far away.

A very, huge and special thank goes to Luisa and to the Ashman\Grover family; thank you for your love, friendship and for the fact I always felt at home. A big portion of this work happened because of you and to you goes my eternal gratitude. There is no need for words, me and you know it all. You will always be in my heart, no matter what.

A special thank and mention to my friends and band members of Mississippi Riot: Jack, Tom, Will and Tim. Music has always been, is and will always be a part of me and my life; sharing this adventure with you made my life fantastic and I accomplished also because of you, so thank you. To a bright rock future!

I would also like to thank my best friend Pierpaolo and "Il branco": Andrea, Luca, Antonio and Paolino. The thought of having brothers like you makes me feel incredibly lucky. I know you will secretly be proud of me, although you will always prefer my brother. Thank you for being the best people I could ever consider as a friend. See above, me and you know everything needs to be said. A special mention goes to Jake and Omar, who I consider best friends as well; we shared incredible experiences, daily life, laughs and tears. I cannot count how many times you have supported me during this journey and, for this, I am deeply grateful. A very special mention goes to my lovely Ilaria for being incredibly, constantly supportive, especially in my darkest moments. Thank you for always being there, especially when I wanted to give everything up. It's also because of you I am here today.

Last but not least, I would like to thank everyone else who has supported me throughout these years; even the smallest thing did matter to me and made me accomplish this.

ABSTRACT

Tyrosine kinase inhibitors (TKIs) are currently the first therapy option for chronic myeloid leukaemia (CML) and acute myeloid leukaemia (AML) patients. However, many patients affected by CML and AML may develop resistance to TKIs or may not recover under this treatment regime. New potential and more effective treatments are recently emerging. Heat shock protein inhibitors (HSPIs) and the proteasome inhibitor Bortezomib are drugs which have been yet to be successfully tested on leukemic patients, despite being successful on other malignancies such as multiple myeloma (MM). The combination between HSPIs and Bortezomib could potentially be successful in killing leukemic cells, by enhancing their respective molecular mechanisms. Indeed, HSPIs would bind to HSP72 avoiding the protein to exert its ligase function to the proteasome, whilst Bortezomib could stop the ubiquitinated proteins to enter the proteasome and ultimately inducing apoptosis. To test the effects of such combination, cell viability was measured via MTS assay, apoptosis levels were tested through Annexin V\PI assays. Involvement of HSP72 and pro-survival protein Bcl-2 were measured via flow-cytometry. The cells were administered with HSPIs and Bortezomib first as single agents for 24 hours, to establish working minimal concentration. Also, the drugs were tested for a shorter time, to understand when the drugs start to be effective. It emerged that one hour is sufficient for the drugs to give an initial effect in terms of cell viability and apoptosis. Following, combination experiments of HSPIs and Bortezomib were performed; the first drug was administered for one hour, the second following one hour and the cells were incubated for 24 hours. This was repeated alternatively for both type of drugs on the different cell lines. MTS and Annexin V\PI showed that there is not a synergistic effect between drugs, but instead there is antagonism. No necrosis was found at any level of the study. The cells were then probed for HSP72 and Bcl-2, to investigate their involvement in apoptosis mechanisms. Following 6 hours of combined and single agent treatment, both type of drugs inhibit HSP72 but failed to reduce the expression of Bcl-2, particularly on AML cells. It is thus proposed that CML and AML cells may die by apoptosis following a short time of treatment with HSPIs and Bortezomib by an extrinsic pathway of apoptosis, independent from Bcl-2 involvement and from mitochondrial pathway of apoptosis. This study may be the first to indicate a potential use of HSPIs and Bortezomib on CML and AML patients for a short time of treatment, although not in combination. Future studies are needed to further investigate the mechanisms of action of these drugs, aiming to potentially give CML and AML patients another successful therapy option to overcome resistance to canonic chemotherapy.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1

CHAPTER 3

CHAPTER 4

CHAPTER 5

CHAPTER 6

LIST OF TABLES

CHAPTER 1

CHAPTER 3

CHAPTER 4

ABBREVIATIONS

3-MA 3-methyladenine

AIF Apoptosis-Inducing Factor

AKT Protein-kinase B

ALL Acute Lymphocytic Leukaemia

AML Acute Myeloid Leukaemia

APAF1 apoptotic protease activating factor-1

ASR Age Standardised Rate

ATP Adenosine triphosphate

ATRA All-Trans Retinoic Acid

Bcl-2 B-cell lymphoma 2

Bcl-xL B-cell lymphoma-extra large

BCR-ABL Break-Point Cluster - gene Abelson

CARD Caspase Activation and Recruitment Domain

CLL Chronic Lymphoblastic Leukaemia

CLP Common Lymphoid Progenitor

CML Chronic Myeloid Leukaemia

CMP Common Myeloid Progenitor

c-MYC Myelocytomatosis

DISC Death-Inducing Signalling Complex

EGFR Epidermal Growth Factor Receptor

FAB French-American-British

FADD Fas-Associated Protein with Death Domain

FASL Fas ligand

FCR Fludarabine, Cyclophosphamide and Rituximab

FLT3 FMS-like tyrosine kinase 3

GA Geldanamycin

GDP Guanosine diphosphate

GRB2 Growth factor receptor-bound protein 2

GSH Glutathione

GTP Guanosine-5'-triphosphate

HSC Haematopoietic Stem Cells

HSP Heat Shock Protein

HSPIs Heat Shock Protein Inhibitors

IAPs Inhibitor of Apoptosis Proteins

IFN- γ Interferon gamma

IKK-β IκB Kinase

IL-3 Interleukin 3

IκB Inhibitors of Kb

JAK 2 Janus Kinase 2

JNK c-Jun N-terminal kinases

LTC-IC long-term culture-initiating

MAPK Mitogen-Activated Protein Kinase

MOMP Mitochondrial Outer Membrane Permeabilization

MSC Multipotent Stem Cell

MTS 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt

NF-κB Nuclear Factor Kappa-Light-hain-enhancer of activated B cells

PES Phenazine Ethosulfate

PES-CL (2-(3-chlorophenyl) ethynesulfonamide)

PI2 Phosphatidylinositol 4, 5 Bisphosphate

PI3 Phosphatidylinositol 3-Phosphate

PI3K PI 3-kinases

PIAS Protein inhibitor of activated STATs

PIs Proteasome Inhibitors

PP2A Phosphatase Protein Phosphatase 2A

PTP Protein-tyrosine phosphatase

RIP Receptor-interacting protein

ROS Reactive Oxygen Species

RT-PCR Reverse transcriptase-polymerase chain reaction

SET SET nuclear oncogene

SH2 Src homology-2

SOCS Suppressor of Cytokine Signalling Proteins

STAT 3 Signal transducer and activator of transcription 3

STAT 5 Signal transducer and activator of transcription 5

TKD Tyrosine Kinase Domain

TKI Tyrosine Kinase Inhibitors

TNF Tumour Necrosis Factor

TRADD TNFR1-Associated Death Domain Protein

TRAF6 TNF receptor associated factor

TRAIL TNF-Related Apoptosis-Inducing Ligand

TT Targeted therapy

WHO World Health Organization

ZAP70 Zeta-chain-associated protein kinase 70

Dedicated to my angels nonna Alessandra, nonno Mario, zio Guido and Marco.

CHAPTER 1: INTRODUCTION

1.1 Leukaemia incidence and mortality

Leukaemia is a progressive malignant disease with high incidence and the low survival rates. According to the World Health Organization, childhood leukaemia is the most common form of cancer within children; for example, the incidence in the United States is 1.6 per 100.000 population. It has also been classified as one of the 15 top cancers in adults for mortality (Kampen K.R., 2012). Like for almost all types of cancer (excluding prostate and ovarian cancer for obvious reasons), the incidence and mortality are higher in men than female populations. Particularly, the incidence of leukaemia in men populations is of 5.6 % and the relative mortality is of 4.2 %; the incidence in women populations, instead, is of 2.7 % and 3.3 % respectively, as shown on Fig.1.1.1. and Fig.1.1.2. The data are considered as Age Standardised Rate (ASR), therefore they are for all ages (World Health Organisation, 2012 and Bhayat F. *et al*, 2009). The reason of the difference in incidence is not still completely known; a study by (Jackson *et al*., 1999) suggested the presence of a mutated gene near the location of the ABO gene on chromosome 9. The percentage of this mutation was found superior in males with AML than on females with AML; however, this could not be considered a definite proof and needs further investigation (Jackson *et al*., 1999).

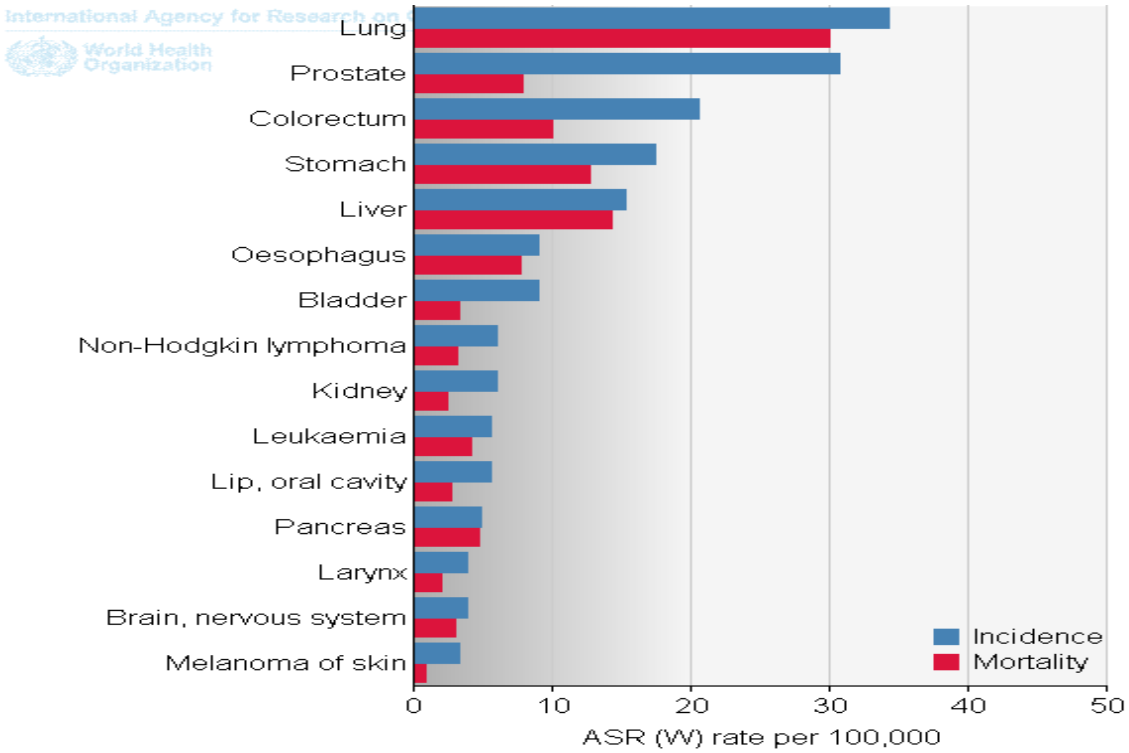


Fig. 1.1.1. The incidence and mortality in men worldwide populations of the most common types of cancer, including leukaemia (retrieved from Globocan, 2012).

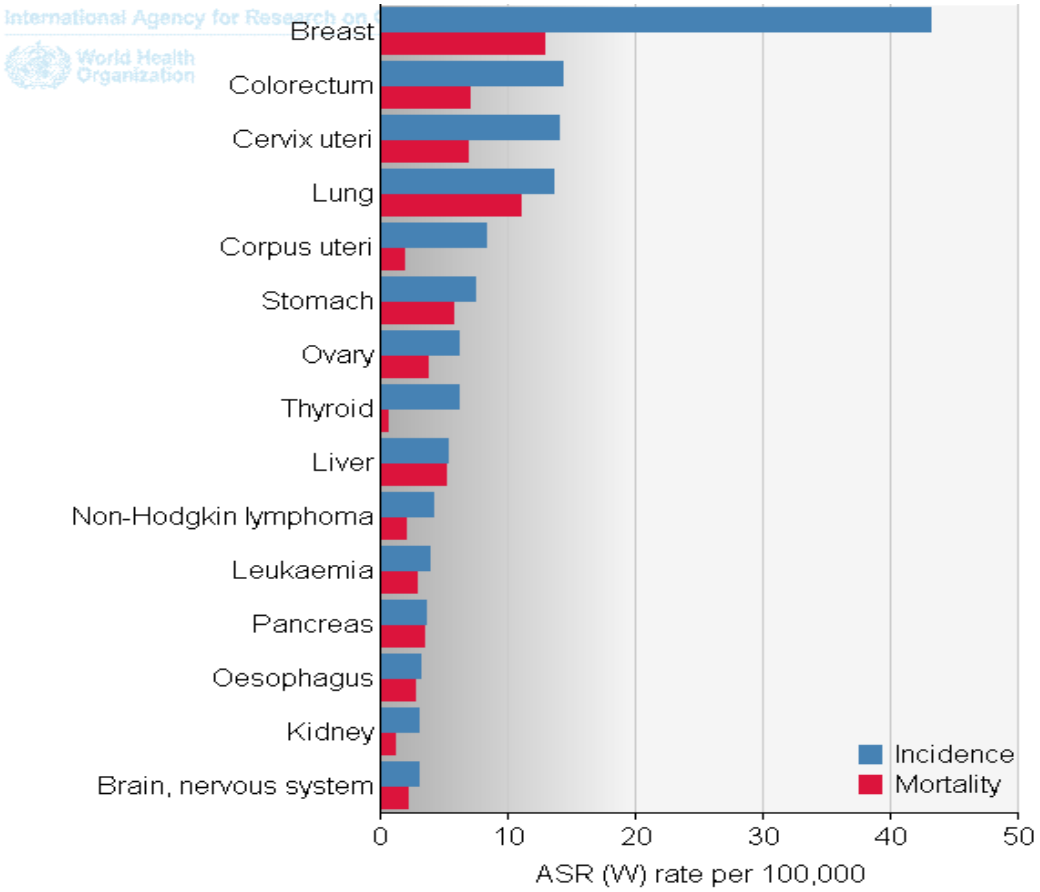


Fig. 1.1.2. The incidence and mortality in women worldwide populations of the most common types of cancer, including leukaemia (retrieved from Globocan, 2012).

With respect of UK, Leukaemia is the 8th most common cancer for men and the 9th for women; the incidence and mortality are higher for both sexes compared to the worldwide populations. Men incidence and mortality are 9.3 % and 4.1 %; women statistics are 5.8 % and 2.4 %, as shown in the graphs below (Fig.1.1.3. and Fig.1.1.4.).

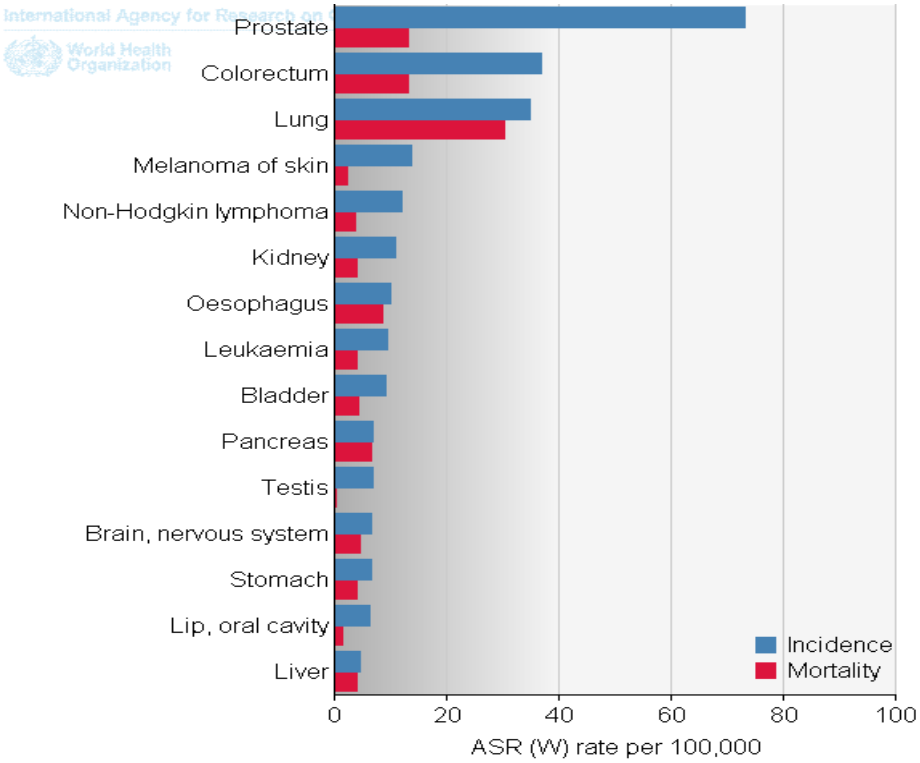


Fig. 1.1.3. The incidence and mortality in men UK populations of the most common types of cancer, including leukaemia (retrieved from Globocan, 2012).

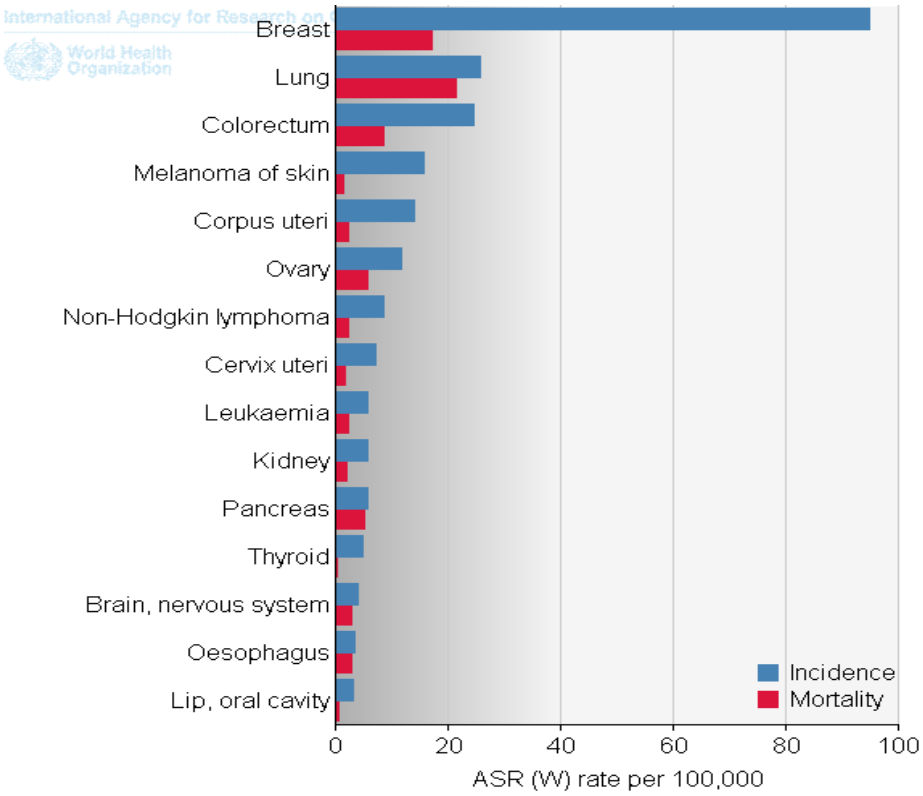


Fig. 1.1.4. The incidence and mortality in women UK populations of the most common types of cancer, including leukaemia (retrieved from Globocan, 2012).

1.2 Haematopoiesis

Haematopoiesis is the process that allows blood cells to form from haematopoietic stem cells (HSC) in the bone marrow. One of the key characteristic of HSC is their ability to self-renew, guaranteeing a constant production; a bone marrow of a healthy adult produces 10 ^{11}new blood cells every day. The formation of new cells is asymmetric and it starts from progenitor cells which differentiate developing two distinct cell lineages: myeloid lineage and lymphoid lineage (Jagannathan-Bogdan, M., & Zon, L. I., 2013) Through further differentiation, the myeloid and lymphoid stem progenitors gradually form the mature blood cells which are erythrocytes, megakaryocytes and myeloblasts for the myeloid lineage; B and T lymphocytes belong to the lymphoid lineage, instead. Megakaryocytes are responsible of the production of platelets, which are fundamental for the homeostasis. Monocytes, eosinophils, neutrophils and basophils derive from myeloblasts. Fig. 1.2.1. sums schematically the haematopoiesis (Ho M.S., *et al.*, 2015).

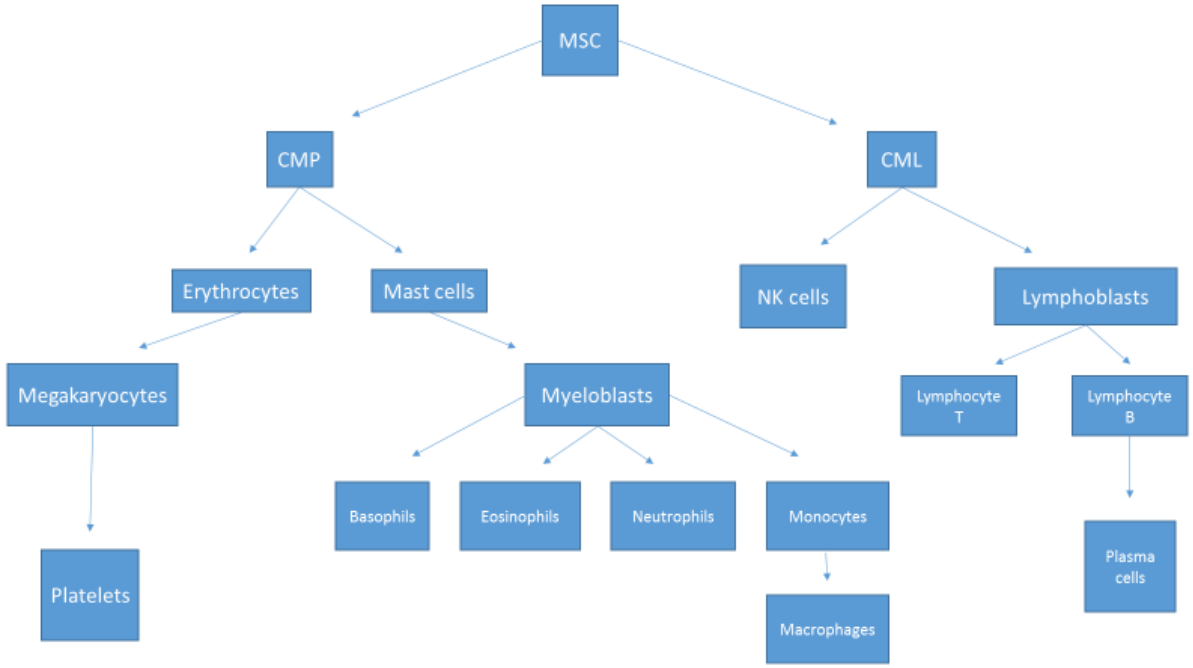


Fig.1.2.1. Haematopoiesis scheme. All blood cells come from a common progenitor, which is the multipotent stem cell (MSC); this stem cell subsequently is divided into common myeloid progenitor (CMP) and common lymphoid progenitor (CLP). From these two, the myeloid and lymphoid lineage will be originated. CMP originates erythrocytes, which produces megakaryocytes. Platelets are therefore produced by megakaryocytes. CMP is also responsible of the production of mast cells, which originate myeloblasts. Basophils, eosinophils, neutrophils and monocytes belong to the myeloblasts family. Monocytes will transform in macrophages. NK cells and lymphoblasts belong to lymphoid lineage originated from CLP. Moreover, lymphoblasts could divide into lymphocyte T and lymphocyte B, which could mature into plasma cells (adapted from Ho M.S., *et al*., 2015 and from Jagannathan-Bogdan, M., & Zon, L. I., 2013)

1.3. Leukaemia: molecular pathology and treatment

Leukaemia, as a broad definition, is generally known as the “blood cancer”; within the disease, there are different types of leukaemia, which share common features and are different from many aspects. The four main types of leukaemia are:

- Chronic Myeloid Leukaemia (CML)
- Acute Myeloid Leukaemia (AML)
- Chronic Lymphoblastic Leukaemia (CLL)
- Acute Lymphocytic Leukaemia (ALL)

The term “chronic” refers to a persistent condition, whilst “myeloid” describes the cell lineage involved. Similarly, “acute” stands for a more severe and rapid course of the disease; “lympho” indicates that the lymphocytes are majorly involved (Colvin G. A. and Elfenbein G. J., 2003). In the below sections, causes of CML and AML are further analysed, given their importante on this thesis. Also, other characteristics such as signs, symptoms and molecular diagnosis are shortly described, for all the types of leukaemia.

1.3.1. Causes of Chronic Myeloid Leukaemia (CML) and Acute Myeloid Leukaemia (AML)

It is a common misconception to attribute a single common cause to Leukaemia; although the different types of Leukaemia have many similar features, they have different causes. With respect of Chronic Myeloid Leukaemia (CML), it is believed that a genetic chromosomic mutation is involved in the occurring of the disease. In 1960, Newell and Hungerford discovered an abnormal small chromosome in the leukocytes from CML patients; this unusual chromosome was not present on normal leukocytes. The newly discovered chromosome was called Philadelphia chromosome because of the location of where Newell and Hungerford were working. Several years following the first identification, it was possible to determine that the Philadelphia chromosome was the result of a reciprocal translocation between chromosomes 9 and 22 (q34; q11); consequently, the genes Abelson (ABL) and break-point cluster (BCR) were fused (Koretzky G.A., 2007). Thus, the product is a mutated protein, BCR-ABL, that is deeply involved in the altered hematopoiesis. Other cytogenetic abnormalities could occur, following the course of the disease; some of them could also lead to treatment resistance, which is constantly object of research and studies (Hanlon, K. & Copland, M, 2017). Fig. 1.3.1.1. shows how the Philadelphia chromosome is formed.

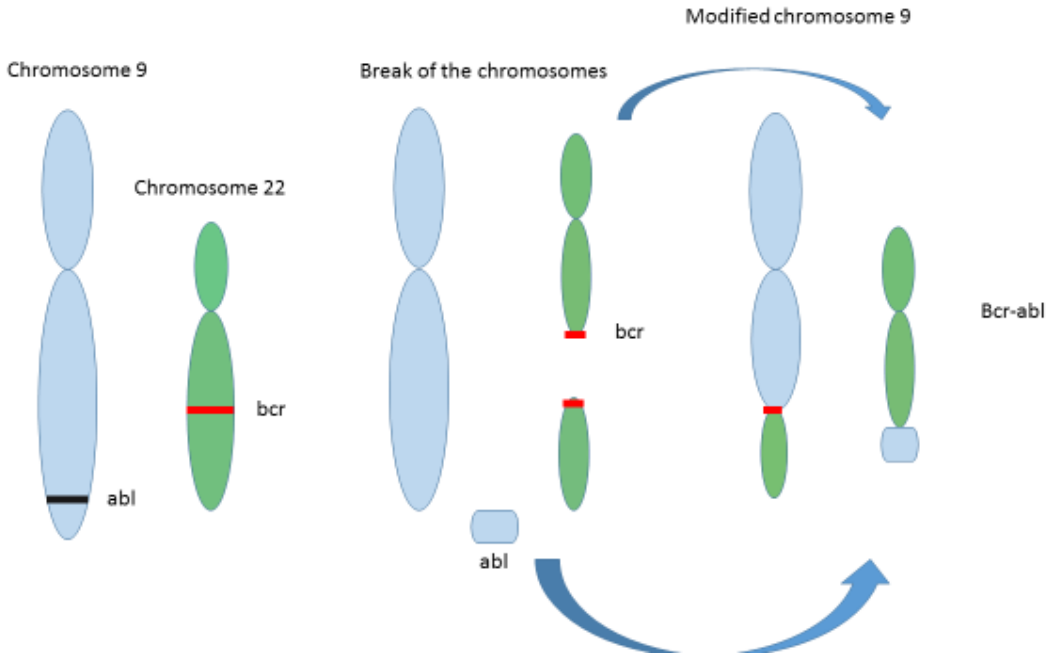


Fig. 1.3.1.1. The creation of Philadelphia chromosome. After a chromosomes break, the genes BCR and ABL merge together forming a small mutated chromosome and, therefore, an oncoprotein (adapted from www.cancer.gov).

AML has an important genetic cause too; one third of the patients diagnosed with AML has a modified gene called FLT3. Within the FLT3 gene, three different mutations have been discovered. The most common mutation is FLT3\ITD, which affects around 20 % of the patients with FLT3 mutation. The genetic modification causes an exponential cell growth, following the constitutive activation of the tyrosine kinase domain. Less frequent are FLT3\TKD mutations and mutations on the juxtamembrane domain (Kayser S. and Levis M.J., 2014). A study by Schnittger S. *et al*, (2002) screened 1003 patients with AML to further investigate the frequency of FLT3 mutation; the length mutation was found on 234 patients (23 %), resulting to be the most frequent of the aberrant mutations. Another study by Kiyoi H. *et al*. (1999) has individuated 43 patients with FLT3 mutation in a cohort of 201; interestingly, 25 patients presented the N-RAS gene mutation, which is a secondary genetic cause of AML. Indeed, although less frequent than FLT3 mutation, patients with N-RAS showed to have lower complete remission than patients with FLT3; therefore, the knowledge of which type of mutation an AML patient has could be an important prognostic marker and could play an important role in the treatment.

1.3.2. Chronic Myeloid Leukaemia: signs, symptoms and molecular diagnosis

The main symptoms and signs could be fatigue, the loss of weight, splenomegaly, leukocytosis, severe anaemia, bleeding, purpura and thrombocytosis (Faderl S*., et al*., 1999). After a mild chronic phase, about 85 % of patients with CML has a progression to an accelerated and more severe phase. Accelerated phase generally leads to the finale stage of the disease which is called blast crisis; it normally arrives to this stage within 6 months (Druker B.J. *et al*, 2001). A main feature of the accelerated phase is the presence of 15-30 % of blasts in marrow or peripheral blood. Also, the thrombocytopenia must be less than 100×10^9/L, referring to platelet counts (Talpaz M. *et al*, 2002). A DNA analysis through PCR technique is one of the main investigation that aims to diagnose CML; the eventual presence of a Philadelphia chromosome indicates a very high risk of having the disease. Also, full blood count and bone marrow biopsy have shown to be important in the diagnosis process, particularly in investigating the severity of angiogenesis in patients (Aguayo A. *et al*, 2000).

1.3.3. Acute Myeloid Leukaemia: signs, symptoms and molecular diagnosis

Similarly to CML, there is a strong genetic component in the causes of AML; a frequent chromosome abnormalities on FLT3 and KIT genes are considered one the most relevant causes of the disease. The prognostic relevance of FLT3 mutation has been evaluated in several studies; for example, Whitman *et al*. have found FLT3 mutated gene in 23 patients out of 82 (28%). The samples taken into consideration were bone marrow samples and they had normal cytogenetics structures (Whitman S.P. *et al*, 2001). FLT3 mutations could be of two types: a tandem duplication or a point mutation. The tandem duplication occurs near the juxtamembrane domain of the receptor (FLT3\ITD mutation) and they are present in the majority of the cases. Instead, the point mutation is less frequent (7 % of the patients) and it is located within the activation loop of the tyrosine kinase domains (FLT3\TKD). Having two different kind of mutations lead to different biological and clinical effects, also due to different mechanisms of activation. The juxtamembrane region where FLT3\ITD is found, has a constitutive and aberrant signalling mechanism caused by its autoregolatory function; therefore, duplicate of tyrosine residues could be added. This could possibly result in more severe prognosis (Levis M., 2013). In order to diagnose and to treat the AML patients, classifications of AML sub-types have been made. The most consulted are the World Health Organization (WHO) and the French-American-British (FAB) ones; WHO have differentiated the sub-types considering the difference in chromosomal abnormalities, whilst the FAB consider the type of cell and the maturity stage at the moment of the diagnosis. Table 1.3.3.1. indicates the lists according to the two main classifications.

SUB-TYPE ACCORDING TO W.H.O.	MAIN CHARACTERISTIC	SUB-TYPE ACCORDING TO F.A.B.	MAIN CHARACTERISTIC
Acute myeloid leukemia with recurrent genetic abnormalities	Mutations on RUNX1-RUNX1T1 or CBFB-MYH11.	M0: Myeloblastic without differentiation	Minimal differentiation, agranular blasts
AML with myelodysplasia-related changes	Presence of 50% or more dysplastic cells	M1: Myeloblastic with little or no maturation	Frequent basophilic cytoplasm, minimal differentiation, blasts are less than 10 %
Therapy-related myeloid neoplasms	Development of neoplasms following cytoxic therapy	M2: Myeloblastic with maturation	Frequent basophilic cytoplasm, start of differentiation: promyelocites\myelocytes
Myeloid sarcoma	Relapse from AML or progression of prior MDS	M3: Promyelocytic	Large granuoles, Auer rods
Myeloid proliferations related to Down syndrome	Transient abnormal myelopoiesis, mutation on GATA1 gene and on JAK-STAT	M4: Myelomonocytic	Abundant cytoplasm, one or more nucleoli, lacy chromatin
Blastic plasmacytoid dendritic cell neoplasm	Clonal proliferation of plasmacytoid monocytes	M4eo: Myelomonocytic with eosinophils	Abundant cytoplasm, one or more nucleoli, lacy chromatin, increase in eosinophils number
AML not otherwise categorized	Less than 20 % of blasts in the bone marrow	M5a: Monocytic without differentiation (monoblastic)	Rough circular nucleus, lacy chromatin, abundant cytoplasm,
		M5b: Monocytic with differentiation	More convolute nucleus, metachromatic granules
		M6: Erythroleukemic	Erythrocytes with multinuclei, cytoplasmic buds, cytoplasmic pseudopods.
		M7: Megakaryocytic	Medium to large sized megakaryoblasts with round nucleus, agrunalar cytoplasm, similarity to lymphoblasts

Table 1.3.3.1. List of sub-types of AML according to WHO and FAB classification.

Grouping the sub-types is enormously important for the diagnosis and the subsequent treatment, although some treatment is in common between various sub-types. The treatment will be further described on section 1.6.

To diagnose AML, the presence of Auer rods it is incredibly helpful. Auer rods are cylindrical crystallized granules of lysosome material visible in a patient smear. Their presence it is a common feature of immature myeloblasts, so it is more likely to find them in the first stages of the disease (e.g. M0 to M4) (Mohamed, M., Dun, K., & Grabek, J., 2013). Fig.1.3.4.1. shows the presence of Auer rods on an AML smear.

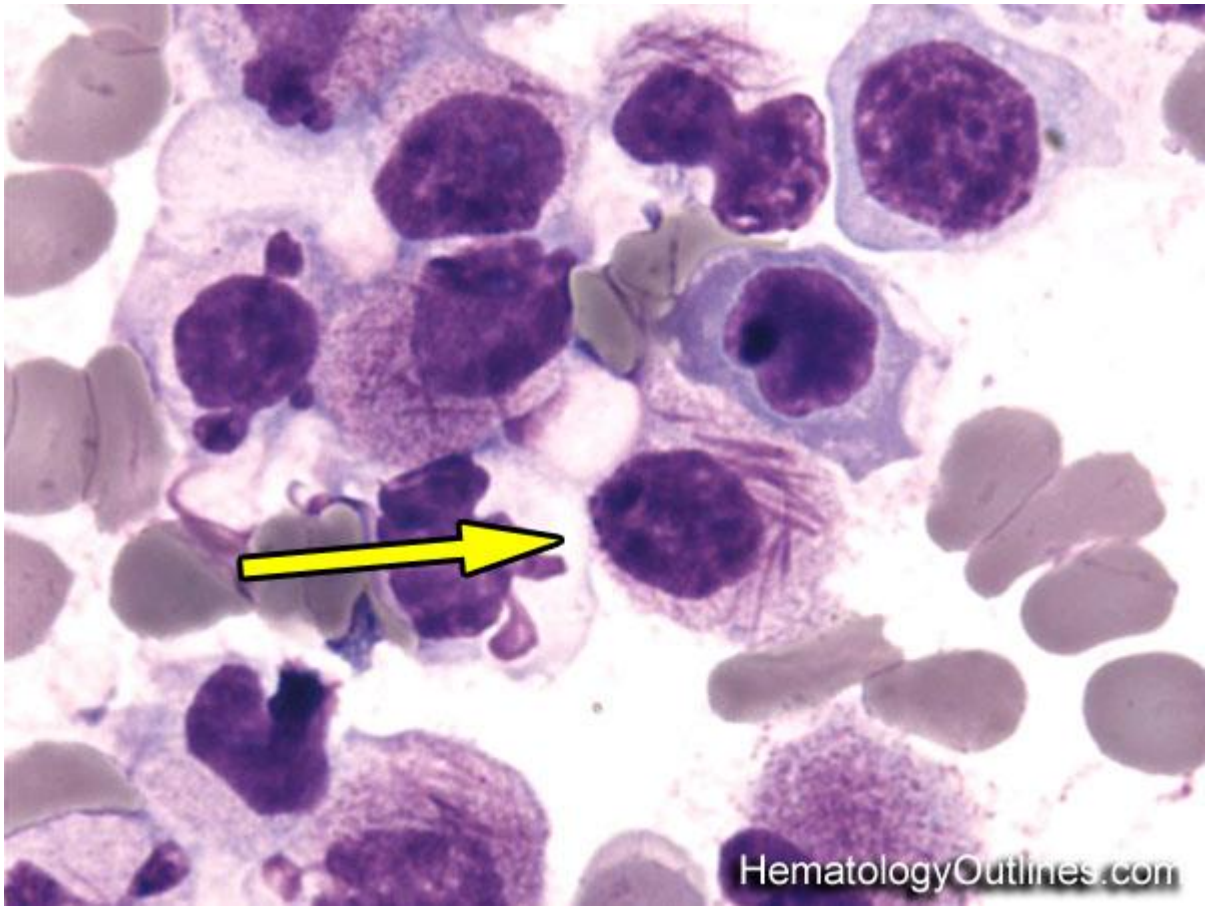


Fig 1.3.3.1. Auer rods in an AML smear. The image clearly shows the formation of granules and, mostly, the presence of Auer rods in the cytoplasm of an immature leukocyte (retrieved from www.hematologyoutlines.com)

1.3.4. Chronic Lymphoblastic Leukaemia

CLL, also known as chronic lymphocytic leukaemia, is the most common type of leukaemia. It affects, as the name suggests, the lymphocytic B cells. To determine the occurrence of CLL, the number of leukaemic cells must be more than 5000 in a uL of blood; also, the cells must have a specific expression of CD19+, CD5+, CD23+ (Weistner A., 2015). B cells are activated also through the recruitment of T cells, which causes the development of memory B cells. With respect of genetic abnormalities, the presence of a mutation on genes V and the expression of CD38 and ZAP-70 intracellular signal protein could help with the treatment options; it has been demonstrated that the CLL cases with a poor level of mutations on V genes and with a high expression of CD38 and ZAP-70 has a much aggressive and often fatal outcome. On the contrary, patients with mutated V genes and few cells expressing CD38 and ZAP-70 had a milder disease progression (Oscier D.G., *et al*., 2000 and Chiorazzi, *et al*, 2005., and Ferrarini, M., M.D, 2005). CLL seems one of the most curable type of leukaemia, according to (Hallek M. *et al*., 2010). After treating CLL patients with a consolidate treatment options named FCR (fludarabine, cyclophosphamide and rituximab), the complete remission was found on 72 % patients; also, the survival rate was more than five years after treatment (Hallek M.*et al*, 2010 & Thompson P.A. *et al*, 2016).

1.3.5. Acute lymphocytic leukaemia

ALL is a type of leukaemia that affects mostly children and\or young people. According to statistic data, about 60 % of the patients is younger than 20 years; therefore, this type is also known as Childhood acute lymphocytic leukaemia. ALL has a high rate of survival; in a study among 21.626 patients in US, 83.7 % had a 5 years survival rate (Hunger S.P *et al*, 2012). Studies on ALL pathobiology have highlighted several chromosomal abnormalities; most of the cases have hyperdiploid mutations on chromosomes 4, 6, 10, 14, 17, 18, and 21 (Inaba, H., Greaves, M., and Mullighan, C. G., 2013). Cytogenetic analysis helps the detection of chromosomal abnormalities; after decades of studies, it is possible to determine that the most common mutations happen on TEL-AML1, MLL-AF4, E2A-PBX and, interestingly, also on BCR-ABL genes. After a RT-PCR analysis, (Pakakasama, S., *et al*., 2008) declared that 25.4 % of patients on their studies resulted with a TEL-AML1 mutation, suggesting that it is the most common chromosome abnormality in ALL among 65 cases considerated (Pakakasama, S., *et al*., 2008). Fig.1.3.6.1. shows a blood smear of an ALL patient.

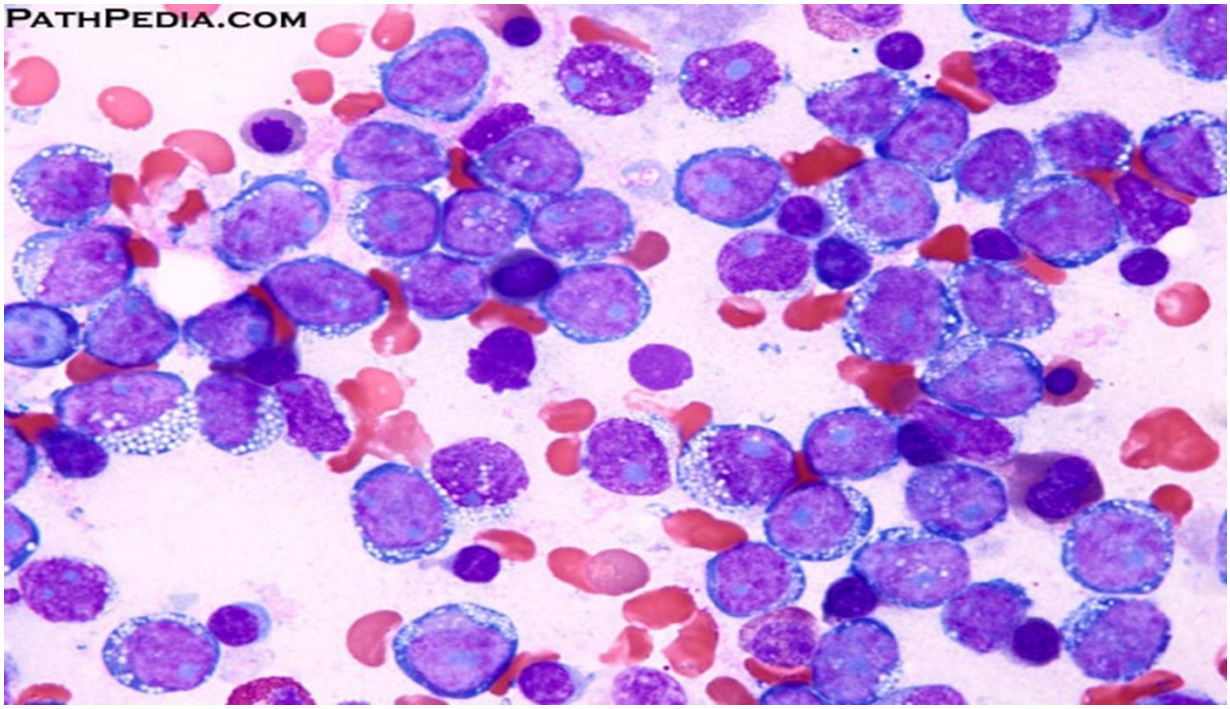


Fig. 1.3.5.1. A blood smear of an ALL patient. The lymphocytes vary in size and they show an abundant presence of cytoplasmic vacuoles, an important feature of acute leukaemia (retrieved from www.pathpedia.com)

With respect of treatment, it is normally divided into three major steps that are remission-induction therapy, consolidation and continuation (Inaba, H., *et al*., 2013). During the first phase, prednisone and dexhamethasone are widely used showing promising results (Pui, C. H., *et al*., 2012). The therapy then is consolidated with methotrexate, vincristine and imatinib (if the patient has a BCR-ABL protein activated); the re-induction therapy could last up to 5-7 months and the drugs are administered weekly. This step is significant mostly because it aims to eliminate the residual leukaemic cells (de Labarthe A. *et al*, 2007 and Stanulla M. and Schrappe M., 2009). The consolidation therapy, as final step, could last up to 2 years from the start of the therapy. Dexamethasone is normally administered, together with mercaptopurine which is preferred to tioguanine, a similar but less efficient drug. Considering that about 20 % of patients of ALL will have a relapse, the consolidation therapy becomes extremely important (Coustain-Smith E. *et al*, 2000).

To summarize the difference in genetic mutations and abnormalities which may cause different types of leukaemia, a table has been drawn (Table 1.3.5.1.).

TYPES OF LEUKAEMIA	MUTATED GENES
CML	BCR-ABL
AML	FLT3, KIT
CLL	GENE V
ALL	TEL-AML1, MLL-AF4, E2A-PBX

Table 1.3.5.1. Summary of genetic abnormalities for all the types of leukaemia.

1.4. Cell death

Cell death is a natural and fundamental step in cell life; at the end of their life spans or due to internal injury, the cells need to choose how they could die. Their destiny is strictly regulated and it could occur in many ways, which are separate and distinct in respect of different pathways and morphology. The recognised types of cell death that will be discussed in the thesis are apoptosis and necrosis.

1.4.1. Apoptosis

The death of a cell could occur in several ways and with different mechanisms; one of them is apoptosis or programmed cell death. At the end of their lifespan or to maintain homeostasis, cells could undergo apoptosis, which suggests that it is a normal process related to aging and cell development. Apoptosis could occur also after cell damages or in response of signals induced by the immune system (Elmore S., 2007). The cells that undergo apoptosis are characterized by an initial shrinkage which results in a condensed chromatin, which is possible to clearly distinguish at the electronic microscope. The apoptotic cells have a rounded shape and they are also morphologically featured by separated cell fragments extruded by the cell by an event called budding; the cell fragments, also called apoptotic bodies will be phagocytized by macrophages (Saraste A. and Pulkki K., 1999). These can be also considered as early apoptotic cells; also, cells could go into a late apoptosis state. If a cell has an impairment of phagocytosis, the cell membrane becomes more permeable and the cells go into a state which is often wrongly considered similar to necrosis. Indeed, the late apoptosis cells releases a permeabilized cell-derived factor outside the membrane which is not found on necrotic cells, for example Poon, I. K. H., Hulett, M. D., & Parish, C. R. (2010). This is recognized by the macrophages which will engulf the cell. Early apoptosis is distinguished into two main types which have separate pathways; there is an extrinsic and intrinsic pathway.

Intrinsic pathway: following stimuli such as hypoxia and radiation, the mitochondria membrane releases pro-apoptotic proteins into the membrane. In the p53 section, the role of mitochondria in apoptosis is described. Here, it seemed appropriate to describe the role of caspases in apoptosis. Caspases play an important role in maintaining homeostasis by regulating inflammation and, of course, apoptosis. Particularly, they are commonly divided as initiator caspases (caspase−8 and −9) and executioner caspases (caspase−3, −6, and −7), according to their role. Cytochrome- c is released from mitochondria following a stress signal and Apaf-1 binds to it; the nucleotide deoxy-ATP binds to Apaf-1 binding site, causing a conformational change that induces the enzyme CARD to bind. This enzyme is responsible for the recruitment of pro-caspase 9. In total, seven Apf-1 monomers are assembled together with cytochrome c and pro-caspase 9 they form a protein complex called “apoptosome” which activates caspase 9 (Cain K., Bratton S.B., Cohen G.M., 2002). The following step is the activation of caspase 3, 6 and 7 which is the trigger for the membrane shrinking and the apoptosis. The caspase 3 activation is helped by mitochondrial proteins Smac\DIABLO, which block the inhibitor of apoptosis proteins (IAPs), allowing the correct continuation of the apoptotic process (Fulda S. and Debatin K.M., 2006 and Elmore S., 2007). Section 1.21. further describes this pathway and p53 activity.

Extrinsic pathway: Differently from the intrinsic pathway, there is no mitochondria involving. Extrinsic pathway begins with ligand binding to death receptors such as Tumour Necrosis Factor (TNF), TNF-Related Apoptosis-Inducing Ligand (TRAIL) and FASL (Fas ligand); the signal cascade continues with the binding to the receptor cytoplasmic domain of adapter proteins FADD, TRADD and RIP (Ashkenazi, A., 2008). Pro-caspase 8 is then activated, stimulating the formation of Death-Inducing Signaling Complex (DISC). At this stage, caspase 8 is active and this allows the recruitment of pro-caspase 3 and the start of the apoptotic process mentioned above (Fulda S. and Debatin K.M., 2006 and Elmore S., 2007).

1.4.2. Necrosis

Contrary to apoptosis, necrosis is not considered an organised form of cell death; the causes of necrosis have a more traumatic, toxic or inflammatory origin. One of the main feature of necrotic cells is the swollen morphology, which then leads to the formation of blebs; the following event, called pyknosis, is the shrinkage of chromatin in the nucleus. The following event is the fragmentation of the nucleus, also known as karyorrhexis. The destiny of the nucleus is then to dissolve in the cytoplasm. Necrosis can be caused by multiple component of the immune system, such as macrophages and the complement system. Also, pathogens and toxins play a role in the induction of necrosis (Fink, S. L., & Cookson, B. T., 2005). However, recent studies seem to demonstrate that necrosis is indeed regulated, due to the presence of kinase cascades that normally feature apoptotic processes. Also, the anti-apoptotic proteins such as Bcl-2, HSP are equally effective in necrosis. Inflammation and necrosis are strictly linked, in fact necrosis stimulates a host inflammation response caused by the release into the extracellular space from the nucleus of nuclear factors such as HMGB1 (Rock K.L. and Kono H., 2008). The effects of necrosis are toxic and dangerous for healthy cells; the release of chromatin or toxic material may affect healthy cells near to the necrotic cells, expanding the area of necrosis. With respect of cancer, an excess of necrosis due to toxic treatment worsen the condition over stimulating the inflammatory process (Proskuryakov S.Y. and Gabai V.L., 2010). A study by Scaffidi P., Misteli T. and Bianchi M.E., (2002) demonstrated that necrotic cells release a protein called High mobility group 1 (HMGB1), which acts as a chromatin binding factor in the nucleus. The release of HMGB1 strongly induces inflammation; indeed, this protein is also produced by macrophages and activated monocytes and could affect neighbour cells. Apoptotic cells do not release this protein and cytotoxic material in case of cell death, not resulting toxic for the human body. For this reason, the therapy must be as selective as possible and it should aim to induce apoptosis to cells, in order to not affect normal and healthy cells of patients as it could happen in case of necrosis.

1.5. Targeted therapy

In the last decades a new therapy option has successfully emerged, showing constant and increasing results: the targeted therapy (TT). As the name may suggest, this option aims to be extremely specific and effective to a target, which is a tumour cell or a molecule vital for the development of the disease (Sledge Jr, G. W., 2005). The TT also attempts to eliminate the issue relative to a not selective action of chemotherapy, where in fact every cell, regardless of their clinical importance, are killed. However, chemotherapy is still a strong therapeutic option nowadays and it is often combined with TT in all the types of cancer (Flaherty K.T., 2006 and Gampenrieder S.*Pet al*., 2013).

There are two main different approaches with respect of TT: direct and indirect approaches. A direct approach aims to alter and damage the tumour proliferation through the monoclonal antibodies or small molecules (e.g. Tyrosine kinases inhibitors); these drugs bind to specific targets involved in the tumour proliferation and development. The indirect approaches, instead, are based on a "homing" action; the drugs (mostly a monoclonal anti*bo*dy) bind to specific receptors on the antigen surface, penetrating in the cancer cell (Schrama, D., *et al*., 2006). There are different families of drugs that belong to the TT option. The drugs used in this study belong all to the direct approach; particularly, they are small molecules that enter the cell and disrupt their activity causing cell death. Instead a monoclonal antibody could interact with a receptor on the surface of the cell, causing a cascade of events that leads to apoptosis. Therefore, although the aim is the same (cell death), how this is accomplished is different. In leukaemia, especially for CML patients, the most effective and widely used monoclonal antibody is Imatinib; its mechanism of action is described on section 1.6.1. The mechanisms of the drug used for this study are also described in the following sections.

1.5. Current treatment guidelines for CML and AML

Currently, the available treatments may vary according to the type of leukaemia or the stage of the disease. Generally, the guidelines provided by the NICE (National Institute for health and Care Excellence), suggest the administration of tyrosine kinase inhibitors such as Imatinib, Dasatinib, Bosutinib, Nolutinib and Ponatinib for patients with CML. Imatinib is the first drug ever developed to specifically target the BCR-ABL gene; the others are of second generation; therefore these drugs are used only if Imatinib has not given effective results or the patients have developed resistance to Imatinib (Sanford D., *et al*., 2015 and Bitencourt R. *et al*, 2011). With respect to AML patients, stem cells transplant is the first option suggested by NICE; however, if patients are not eligible for such therapy, the first recommended drug is azacytidine. Indeed, it seems that Imatinib, for example is not effective on AML patients (Cortes J. *et al*, 2003 and Burchert A*. et al*, 2005).

However, there are patients who may not be eligible either for stem cells transplant or maybe who are resistant to canonic treatments. Therefore, this gap of new potential therapies could be met by the use of other alternative drugs, which may target different proteins than BCR-ABL or FLT3, on CML and AML respectively. Some of the new potential therapy options have been tested and the results have been shown on this thesis. The following paragraphs describe the mechanism of action of Imatinib and the new potential therapy options tested on this thesis.

1.6. Tyrosine Kinase Inhibitors (TKI)

TKI is a family of drug which aims to inhibit the tyrosine kinases action. Tyrosine kinases are enzymes with a crucial role in signal transmission, due to their ability to phosphorylate residues through ATP. As a consequence, tyrosine kinase could be intended as an “on and off” switching enzyme (Paul, M. K., & Mukhopadhyay, A. K., 2004). A tyrosine kinase receptor has a high affinity to several ligands, such as cytokines and growth factors; once the ligand has bound to the receptor, the cytoplasmatic region transfer a phosphate to the tyrosine residues through the donor, an ATP molecule. Therefore, binding sites are made for signalling proteins having Src homology-2 (SH2) and protein tyrosine binding (PTB) domains; the signalling cascade which will lead to proliferation and cell survival could start (Hubbard, S. R., & Miller, W. T., 2007). In normal conditions, the kinases are strictly regulated; in case of a mutation event, such as the BCR-ABL protein, the phosphorylation activity is modified and incredibly enhanced. Thus, TKI have been developed and launched on the market, gaining an incredible and effective success as a new and, nowadays, consolidate therapy option together with chemotherapy (Arora A. and Scholar E.M., 2005).

1.6.1. IMATINIB

The first TKI available on market in 2001 was Imatinib (Gleevec). It had a revolutionary effect on the current (at that time) therapies and it is still widely used today as a monotherapy or in combination with other drugs (Deininger M., Buchdunger E. and Druker B.J., 2005 & Chalandon Y. *et al*, 2015). The mechanism of action of the drug has a switch on\off concept, given by his high affinity for the tyrosine residues on the cytosolic part of the tyrosine receptor. As mentioned previously, ATP binds to the receptor in order to phosphorylate the substrate; Imatinib occupies the ATP binding site on the BCR\ABL protein, preventing the phosphorylation and, therefore, the start of the cell proliferation (Fig.1.6.1.1.). Also, the inhibition of the kinase stimulates its entry in the nucleus, where all its antiapoptotic features are repressed.

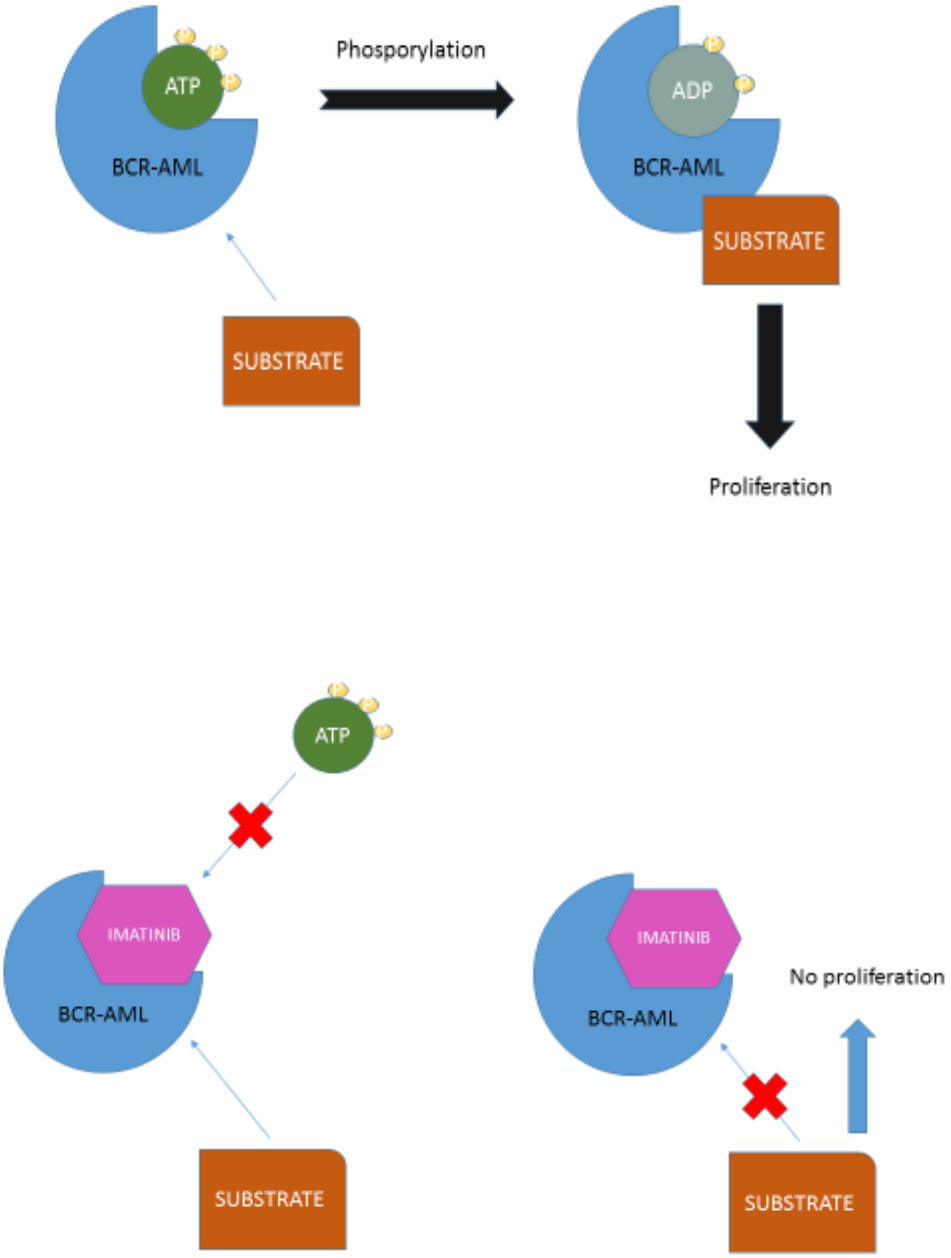


Fig 1.6.1.1. Imatinib mechanism of action: in the upper part it is described how the phosphorylation happens in leukemic cells, in which the substrate binds to the protein leading to cell proliferation. The second part of the figure shows how Imatinib binds to the ATP sites, avoiding the substrate to start its signalling pathway (adapted from Deininger M., *et al*., 2005 & Chalandon Y. *et al*, 2015).

Imatinib has shown an initial incredible enthusiasm due to its effectiveness in vitro and in vivo on patients with Philadelphia chromosome positive following its launch on market (Sawyers C.L. *et al*, 2002). Indeed, one of the cell line used in this thesis (K562) carries the mutated BCR-ABL gene, main cause of CML. Imatinib has been successfully used as treatment not only on leukaemia patients, but also on cases with different tumours, such as gastrointestinal stromal tumours and nephrogenic systemic fibrosis (Huse D.M., *et al*., 2007 & Kay J. and High W.A., 2008). Despite its enormous success, Imatinib it is not effective on every patient; in the years following the first encouraging results, several studies highlighted cases of resistance to the first TKI. The resistance is caused by genetic mutations on the BCR-ABL kinase domains. For example, (Shah *et al*., 2002) have found that on 29 of 32 patients (90 %) with Imatinib resistance had mutations on the kinase domains (Shah N.P., *et al*., 2002). Also, Imatinib seemed to not be particularly effective during blast crisis, despite comforting results on the initial phases of the disease (Goss V.L. *et al*., 2006). K562 cells represent a patient on blast crisis (as further explained on Chapter 2.3.1.); U937 cells, instead, do not express BCR-ABL gene, but seemed to be resistant to Imatinib therapy (Goss V.L. *et al*., 2006 and Hakansson P. *et al*, 2004). Thus, new therapies are needed to overcome drug resistance and potentially give CML and AML patients another therapeutic option to Imatinib; sections 1.9. and 1.14. show some of the new potential therapeutic options for such patients.

1.7. The proteasome

A protein, before becoming active and reaching its target, needs to be “checked” by some structures in the cell; this event consists in the elimination and degradation of damaged or unneeded cells. A proteasome is a multisubunit complex enzyme that plays an important role in this respect. Not all the proteins are destined to be degraded, of course. The misfolded or damaged proteins will enter the proteasome complex only if they have ubiquitin, which is a small protein that is attached to the unneeded protein in order to be recognized by the proteasome complex (Adams J., 2003). The series of event that lead to the attachment of ubiquitin is the ubiquitination. The protein targeted is recognised by a series of enzymes called E1, E2 and E3; E1 transfers the ubiquitin molecule to E2 which binds to E3 and carries ubiquitin to the protein that needs to be recognised by the proteasome. The proteasome complex is also called 26S and it is formed by two subunits, 19S and a central 20S; when the ubiquinated protein enters the proteasome complex, 19S is responsible of breaking the bind between ubiquitin and the protein. Subsequentially, the protein is degraded and the ubiquitin could be re-used for another ubiquitination process (Crawford L.J., Walker B. and Irvine A.E., 2011).

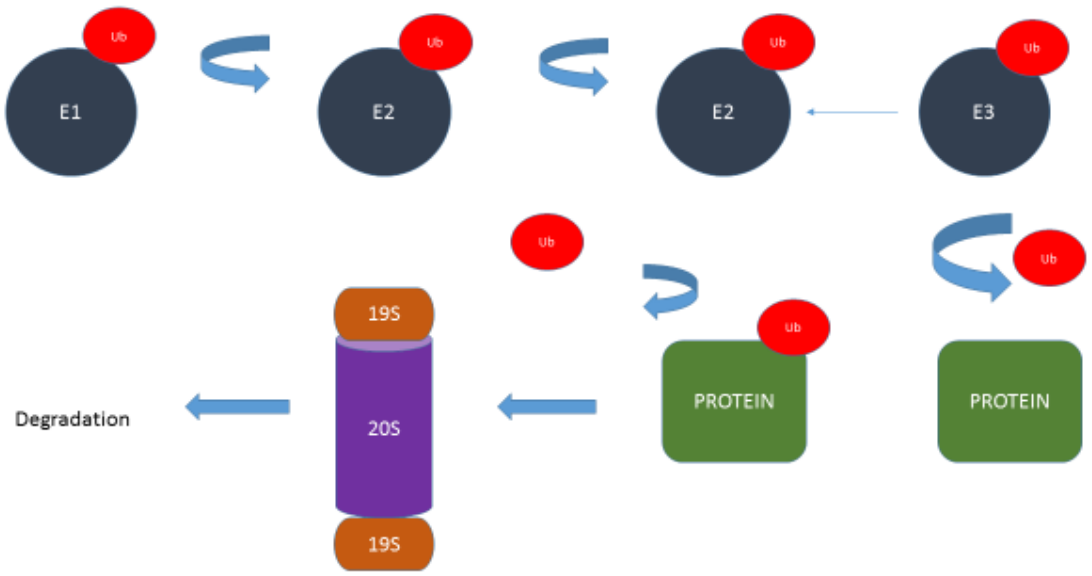


Fig.1.7.1. The ubiquitin-proteasome pathway. Ubiquitin is carried by E1 and E2, which binds to E3 to release Ubiquitin to the protein substrate. The protein faces degradation entering the proteasome complex, composed by a central 20S subunit and two 19S subunits. The ubiquitin molecule is then released to be available for other ubiquitination processes (adapted from Crawford L.J., *et al*., 2011 and Ben-Neriah, Y., 2002).

1.8. Proteasome Inhibitors

In leukemic patients, the proteasome activity is altered causing an uncontrolled survival rate and spread. The proteasome inhibitors (PIs) aim to induce apoptosis by targeting the protein involved in cell growth and leading them to degradation through different mechanisms; one of these is the blockage of the NF-κB pathway that has a crucial role in cell viability (Adams J. *et al*, 1999). Some studies strengthen the importance of PIs in leukaemia treatment; in fact, there is a strong relation between the sensitivity to PIs in leukemic stem cells rather than hematopoietic stem cells as suggested by (Crawford L.J. *et al*, 2009). Also, it has been demonstrated that the proteasome catalytic activity is higher in CML bone marrow cells than control cells, suggesting the importance of PIs in disrupting proliferation cell signalling pathways (Crawford L.J. *et al*, 2009).

1.9. Bortezomib

Bortezomib was first created for Multiple Myeloma patients, reaching interesting results as a single agent therapy, particularly in not previously treated cases (Richardson P.G. *et al*, 2009); the combination with dexhamethasone is also effective and it seems to be a successful option in many studies and trials (Jagannath S. *et al*, 2005 & Harousseau J.L. *et al*, 2010). Bortezomib targets mainly NF-κB pathway, which is a crucial target for the proteasome. IκB is an inhibitory protein which regulates NF-κB by blocking p50 heterodimer to be translocated into the nucleus. Bortezomib inhibits

the 19S subunit, avoiding the inhibitory protein to be degraded and, thus, interrupting the anti-apoptosis cascade in the nucleus. It also has a role in inhibiting angiogenesis and chemotaxis. Moreover, normal cells are less sensible to proapoptotic effects therefore they can rapidly repair their proteasome activity in presence of Bortezomib (Hideshima T. *et al*., 2011). However, some evidence is emerging showing that Bortezomib may not inhibit NF- Kb, but it activates it by down-regulating IκB-α expression in multiple myeloma cell lines and patients. This was demonstrated by the inhibition of IKK-β after treatment with MLN120B (Hideshima T. *et al*, 2009). As previously mentioned, Bortezomib is a widely used treatment in multiple myeloma; however, Bortezomib mechanism of action is not completely clear in myeloid lineages. It has been speculated that Bortezomib could induce apoptosis in AML cells by degrading TRAF6 protein, which belongs to TNF-α family of proteins. The degradation seems to happen through the autophagic induced activity of Bortezomib, which was confirmed by the addition of an autophagy inhibitor, 3-methyladenine (3-MA) to AML cells which then improved the cell viability levels by restoring TRAF6 protein expression (Fang J. *et al*, 2012). K562 cells and U937 cells seem to express mutated TRAF6 protein, among other mutated proteins (Wu Y.H. *et al*., 2018 and Quaranta M.T. *et al*., 2015). Also, it seems that Bortezomib may disrupt the pathway that involves NF-κB and that has as a final target the transactivation of KIT, a tyrosine kinase inhibitor. By modulation of SP1 expression and therefore NF-κB (p65), FLT3 is up-regulated. With respect of CML, bortezomib was also tested in $CD34^{+}$ cells from CML patients. Bortezomib demonstrated to cause apoptosis and inhibit proliferation of CD34+38−, long-term culture-initiating (LTC-IC) cells. Importantly, these patients were previously resistant to treatment including T315I, H396P and M351T (Heaney N.B. *et al*, 2010).

Bortezomib is also involved in p53 regulation; p53 is deeply involved in cell apoptosis and its level is high after a DNA mutation. In case of proteasome disfunction, p53 accumulates into the nucleus, causing an anti-apoptosis effect and promoting cell survival. Indeed, pro-apoptosis proteins such as Bcl-2, Bax and Bak are reduced, causing p53 to not be released in the cytoplasm (Vaseva, A. V., & Moll, U. M., 2009). Further explanation on this mechanism is at Chapter 1.22. Bortezomib induces the rehabilitation of p53 levels and enhances the apoptosis pathway (Ling X., *et al*., 2010). Thus, understanding Bcl-2 involvement following treatment with Bortezomib may possibly improve the knowledge of the apoptosis processes and help understanding Bortezomib effectiveness in inducing apoptosis. Logically, Bcl-2 levels influence protein expressions downstream the apoptosis pathway; therefore it could be considered as a reliable end-point, as further expressed on Chapter 5.

1.10. Other proteasome inhibitors

Following Bortezomib development, other more advanced PIs have been manufactured. MG-132, for example is a synthetic PI used *in vitro* that induces apoptosis through the formation of reactive oxygen species (ROS) and Glutathione (GSH) reducted levels; due to the formation of ROS, the mitochondria membrane is depolarised and releases Cytochrome C. Thus, the oxidative stress is enhanced and the cell viability is compromised, leading to cell programmed death (Guo N. and Peng Z., 2012). There is not sufficient information in regards of MG-132 effects and efficiency, excluding the data that the manufacturers supply, further studies are therefore required.

Another PI available and successful in several studies is carfilzomib, which is a second-generation PI; Carfilzomib showed to be more specific than Bortezomib. The second generation drug is in fact irreversible and it has less chance to involve normal cells and normal functioning proteasome; Carfilzomib has an important effectiveness in patients that received prior unsuccessful therapy, such as Bortezomib or a stem cell transplantation (Stewart, A. K., 2015). With respect to Bortezomib on leukemic cell lines or CML patients, it does not seem to be particularly efficient; instead it showed relatively significant results if combined with dexamethasone, vincristine, pegylated asparaginase and doxorubicin on ALL patients (Messinger Y.H., *et al*., 2012). Therefore, further studies are required in order to determine the reason why Bortezomib is more reliable than second generations PIs with respect to CML patients. Also, further clarifications are needed in order to understand if Bortezomib is effective as a single agent or in combination with other treatment on CML patients, which is what this thesis also aims to prove.

1.11. Heat Shock Proteins

The process of folding a protein is fundamental for the life of any cell. The Heat Shock Proteins (HSPs) are deeply involved in the molecular mechanisms of folding and in the prevention of protein aggregation; they are also recruited in the ubiquitination and proteasome degradation processes. There are several known HSPs and they are classified and named according to their molecular mass or based on the protein that they encode (Calderwood S.K., *et al*., 2006). Therefore, for example, HSP70 or HSP90 are encoding for a protein that has a 70 or 90 kd (kilodaltons). HSP are also called chaperone proteins, because of the role in helping the folding process of the protein. For the purpose of this thesis, HSP90 and HSP70 only will be described, due to the important role in cancer progression and in treatment options. HSP90 is expressed in all eukaryotic cells and HSP90 alpha and beta isoforms are the most abundant in human, also called HSPC1 and HSPC3 (Jego G., *et al*., 2013). With respect to its structure, HSP90 has three main domains: an ATPase N domain, a high affinity co-chaperone and client proteins region and a C- domain (Onuoha S.C., *et al*., 2008). ATP is vital for the correct functioning of HSPs; its binding allows the client protein to bind as well. ATP is then hydrolysed, causing a conformational change from “close” to “open”, so the client protein is correctly released. The regulation of HSPs occurs through more than 20 co-chaperones which generally could inhibit or enhance their activity by assisting the client protein loading or promoting the conformational change, respectively (Honga D.S., *et al*.,2013). In all HSPs, the role of ATP in its regulation and, mostly in its protein loading capacity, is fundamental. However, it is still not clear if ATP hydrolysis is responsible of the release of peptides from the “close” to “open” conformational state (Mahalka, A. K., *et al*.., 2014). However, it is believed that the role of HSP70 as ATP dependent molecular chaperone is to help the process of folding of new proteins, to organize the multi-protein complexes and also to facilitate the transport the proteins across cellular membranes. HSP70 is composed by two separate domains: a peptide binding domain which is responsible for substrate binding and refolding, and the amino-terminal ATPase domain. HSP70 has several co-chaperones that bind to it; one of them is HSP40, which co-stimulate the ATP activity of the protein. Also, another co-chaperone such as BAG-1 binds to HSP70 which then regulates protein like Bcl-2 and RAF-1; the cascade continues with the production of ERK protein, responsible of cell growth and survival (Kliková K., *et al*., 2016). HSPs are abnormally expressed in leukaemia and, more generally, in haematological malignancies. Below are described the implications of HSP90 and HSP70 overexpression in leukaemia.

1.12. HSP90

In cancer patients and, most specifically in leukaemia patients, HSP90 activity is inhibited leading to an ubiquitination process and the resulting degradation in the proteasome; therefore several oncoproteins targeted by HSP90 are overregulated (Flandin-Gresta *et al*., 2012). Automatically, HSP90 has been studied in respect of cancer and leukaemia; the first studies and encouraging results led to the distribution of the first HSP90 inhibitor, geldanamycin (GA) (Jhaveri, K*., et al*., 2012). GA has shown to specifically bind to HSP90, degrading its client protein; moreover, it showed that it restores sensitivity to chemoterapic agents in resistant cell lines (Blagosklonny, M. V. *et al*, 2001). In that respect, an interesting research indicated that GA has an important role in preventing the accumulation of p53 followed the administration of paclitaxel. Also, in a cell-type dependant manner, it triggers apoptosis by activating caspase-3 and caspase-9 in leukaemic cell lines, such as HL-60 and K562 (Nimmanapalli R, *et al*., 2001 & Blagosklonny M.V., 2002). Combination of GA are still object of several studies, aiming to further acquire knowledge on their role in cancer and leukaemia.

1.13. HSP70

Only recently, HSP70 has been indicated as a potential target for cancer cells; a high level of HSP70 is in fact considered a bad prognosis factor (Murphy M.E., 2013). Due to its role in protein homeostasis and in the development of cancer, it is interesting to understand HSP70 structure, because it could suggest how an HSP70 inhibitor binds to the protein, therefore exerting its activity. HSP70 is composed by three major domains: a ~10 kDa C-terminal, a ~15 kDa substrate binding domain and a ~44 kDa N-terminal nucleotide binding domain. The latter one is the site where ATP binds and it is also the site for hydrolysis. The C-terminal works as a "lid"; when the ATP is not binding to HSP70, the lid is closed preventing any interaction with proteins (Schuermann J.P. *et al*, 2008). When the ATP binds to its specific site, a conformational change occurs, opening the lid and increasing the affinity for the substrate binding of a ~10-fold increase. The role of ATP is key in the control and in the function of HSP70, therefore in the understanding of HSP70 role in cancer (Patury S., *et al*., 2009). Interestingly, the mitochondrial isoform of HSP70 has been named "mortalin" due to its over-expression in colorectal, colon and breast cancer cells in mouse models (Dundas S.R., *et al*., 2005). A study by Pocaly M. *et al*, (2007), has demonstrated that over-expression of HSP70 can induce resistance to Imatinib on K562 cells and in CML patients, indicating an important role of HSP70 in the prognosis of CML. However, the role of HSP70 in cancer has not been fully analysed, it is hoped that this thesis could help improving the knowledge on HSP70 involvement with cancer. Some studies have confirmed the specificity of HSP90 inhibitors, such as 17-AAG, to the HSP90 complex, both *in vivo* and *in vitro*. This perhaps suggest that HSP90 could be used as a model for HSP70 specificity experiments, aiming to understand how HSP70 could show selective toxicity as HSP90 has shown to do (Banerji U., 2009 and Kamal A., *et al*., 2003). Belonging to HSP70 family of proteins, the stress-inducible form of HSP70 is known as HSP72 and it is over-expressed in tumours. In case of cellular stress, HSPA1A gene which codes for HSP72 is activated by binding of heat shock factor 1 (HSF-1) to heat shock elements (HSE), located upstream the regulatory regions of HSPA1A. This leads to the over-production of HSP72; on normal conditions, HSP72 levels are very low and HSF-1 does not bind to DNA (Daugaard M., *et al*., 2007). Importantly, although not always specified, this thesis always refers to HSP70 as per HSP72 protein, indicating abnormal conditions like in leukaemia. HSP72 has been found to bind to p53, inhibiting apoptosis and promoting cell survival. Depletion of HSP72,

instead, resulted in improved apoptosis in colon, prostate and breast cancer as demonstrated by (Nylandsted J. *et al*, 2000 and Gabai V.L. et al., 2005). It appears to protect different pro-apoptosis signalling pathways, such as caspase cascades and MAPK pathway, via JNK inhibition. Also, it interacts with Apaf-1, causing apoptosis suppression by blocking the caspase cascade; furthermore, it appears to inhibit caspase independent apoptosis by interacting with apoptosis-inducing factor.

More interestingly, HSP70 inhibitors appeared to contrast other drugs effect when over-regulated, whether it seems to be more effective when it is downregulated (Jego G., *et al*., 2013). HSP72 binds and regulates BCR-ABL activity on K562 cells and in patients with CML blast crisis, has been found. The cell proliferation could be stimulated by upregulation of HSP72 and HSP90 through the control of BCR-ABL tyrosine kinase is stimulated by HSP72 and in turn phosphorylates and activates Akt that inactivates Bad and caspase signalling cascade and phosphorylates STAT5. Phosphorylated STAT5 may be responsible of cell survival by enhancing the expression of anti-apoptotic protein Bcl-xL (Guo F. *et al*, 2005). However, when Imatinib is administered to leukemic cells, BCR-ABL and PI-3K are inhibited, restoring the HSP70 levels and inducing apoptosis. HSP72 has a strong connection with activation of mutant oncoproteins, such as c-Kit or FLT3-ITD, which are responsible of AML poor prognosis (Reikvam H. *et al*, 2012). Also, HSP72 is expressed in other kind of cancer, as extensively demonstrated. High levels of HSP72 have been found on early hepatocellular and prostate cancer; also, in colorectal carcinoma and lung cancer (Murphy M.E., 2013). Surely, its role in cancer and in leukaemia is still not very well known; thus, HSP72 inhibitors and their effectiveness are more likely to be studied in the future, hoping to provide another therapy option for cancer patients.

1.14. HEAT SHOCK PROTEIN INHIBITORS

1.14.1. PIFITHRIN-μ

Pifithrin-μ (Fig.1.14.1.) is a HSP70 inhibitor recently developed, which has a significant antileukemic activity; it selectively interacts with HSP72 inhibiting its chaperone activity. It causes cell cycle arrest and induces apoptosis in AML and ALL cell lines, in a dose-dependent manner. However, it seems to be not similarly effective in respect of CML cell lines and primary cells (Kaiser M. *et al*, 2011). It seems to be efficient against prostate cancer cells, by enhancing hyperthermia; although Pifithrin-μ could induce apoptosis as a single agent, it seems that the combination treatment with hyperthermia improve the effects of the drug. Particularly, it seems that the timing of the drug administration is critical. Pifithrin-μ administered immediately before a heat treatment appears to give the maximum effect; it could be speculated that this drug plays a protective role immediately after heat stress (Sekihara, K., *et al*., 2013). Also, the combination with cisplatin seems to induce apoptosis following 72 hours of treatment. There seems to be a synergistic effect between the two treatments (Mc Keon A. M., *et al*, 2016). Pifithrin-μ also binds to p53 by avoiding the interaction with anti-apoptotic proteins such as Bcl-xL and Bcl2 on the mitochondria membrane; as a result, caspase activation occurs and apoptosis process is triggered (Strom E. *et al*, 2006). Although the mechanism of the translocation of p53 into mitochondria is not completely clear, Pifithrin-μ seems to inhibit the translocation. Consequently, the mitochondria membrane restores its potential, avoiding the release of pro-survival factors. It has been speculated that Pifithrin-μ may be responsible of cell death via a not dependant caspase manner; it seems to bind to apoptosis-inducing factor (AIF), starting the apoptosis process by

not involving caspases. Further, Pifithrin-μ blocks the induced stabilization of lysosome membrane permaibility by binding to HSP70, reducing cell survival rates, a study demonstrated (Nylandsted J. *et al*, 2004). However, the importance and the effectiveness of Pifithrin-μ on leukaemia (particularly on CML) is yet to be completely clear, considering also the lack of literature available currently.

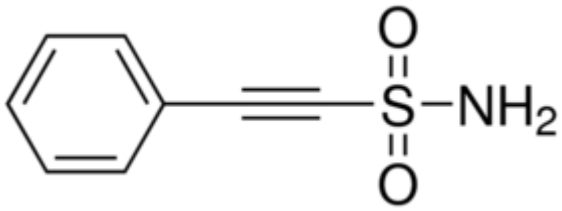


Fig.1.14.1. A molecule of Pifithrin-μ. (retrieved from www.sigmaaldrich.com).

1.14.2. PES-CL

A molecular model revealed binding sites on HSP70 where Pifithrin-μ fails to bind, potentially limiting its effectiveness. Therefore, a derivate of Pifithrin-μ called PES-CL (2-(3-chlorophenyl) ethynesulfonamide), has been recently developed (Fig.1.14.2.). It has been demonstrated that it is more effective on cell viability (measured by MTS assay) than Pifithrin-μ on B-cell lymphoma mice, suggesting a potential therapeutic use on human; also, it targets and leads to inactivation of several HSP90 target proteins, similarly to Pifithrin-μ (Balaburski G.M. *et al*, 2013). PES-CL seems to be more efficient with respect of cytotoxicity of other 3 HSPI, as a study has been indicating; also it is interesting to note that the PES-CL dose which induces a loss of cell viability and induction to apoptosis is less high than the IC50 found on the other HSPI (Budina-Kolomets A., Balaburski G.M., Bondar A, Beeharry N, Yen T. and Murphy M.E., 2014). Similar to Pifithrin-μ, PES-CL molecular mechanisms and effectiveness on CML and more generally on leukaemia, are still not completely understood.

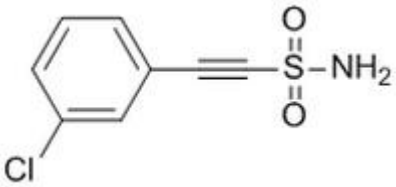


Fig.1.14.2. A molecule of PES-CL (retrieved from www.merckmillipore.com).

Following the description of the diseases and of the drugs, it is paramount to describe in depth the mechanism of the signalling cascades candidate to potentially be inhibited by the drugs above mentioned.

1.15. JAK 2 signalling pathway

The oncoproteins play a fundamental role in the developing and in the synthesis of tumour cells and their exponential growth. Regarding BCR-ABL oncoprotein, it is paramount to understand that it is a protein that has tyrosine kinases effects and linked signalling pathways. One of the kinases majorly involved in the mechanisms of BCR-ABL signalling is the Janus Kinase 2 (JAK 2); through its regulation, BCR-ABL is able to induce leukaemia cells to survive and reproduce constantly. Jak 2 physically binds the C terminal of BCR-ABL, although SH2 domain of BCR-ABL is responsible of the actual phosphorylation of JAK2 on tyrosine residue Y1007 which is required for Jak 2 activation (Xie S., *et al*, 2001). Importantly, Bortezomib appeared to inhibit Jak2 pathway in combination with JAK/STAT pathway inhibitor, called Ruxolitinib (de Oliveira, M. B., *et al*., 2016). The downstream of BCR-ABL is directly related to the interaction between JAK 2 and myelocytomatosis viral oncogene homolog (c-MYC); there are several possible independent mechanisms involved. First, Jak 2 increases c-MYC mRNA levels through the phosphorylation of the v-akt murine thymoma viral oncogene homolog, inactivating the glycogen synthase kinase-3β (Samanta A.K., *et al*., 2009). Also, JAK2 activation results in a high level of c-MYC protein caused by the inhibition of the ubiquitin/26S proteasome; the catalytic activity of Jak2 negatively regulate various pathways including association with Suppressor of Cytokine Signalling Proteins (SOCS), following stimulation by Interleukin 3 (IL-3) and Interferon gamma (IFN- γ) (Warsch W*., et al*., 2002). Finally, the phosphatase protein phosphatase 2A (PP2A) plays a fundamental role in the molecular mechanisms of CML development. PP2A, when active in normal conditions, is responsible of interrupting BCR-ABL signalling pathway through the dephosphorylation of BCR-ABL by a tyrosine phosphatase SHP1. In patients with a modified BCR-ABL protein, PP2A is inhibited by SET nuclear oncogene (SET), a nuclear/cytoplasmic phospho-protein which is present in several solid and haematological diseases. Consequently, Lyn, Jak2, STAT5 pathways are activated, avoiding apoptosis for the leukemic cells (Neviani P,. *et al*, 2005 & Warsch W., *et al*., 2013). Fig.1.15.1 sums the pathway just described.

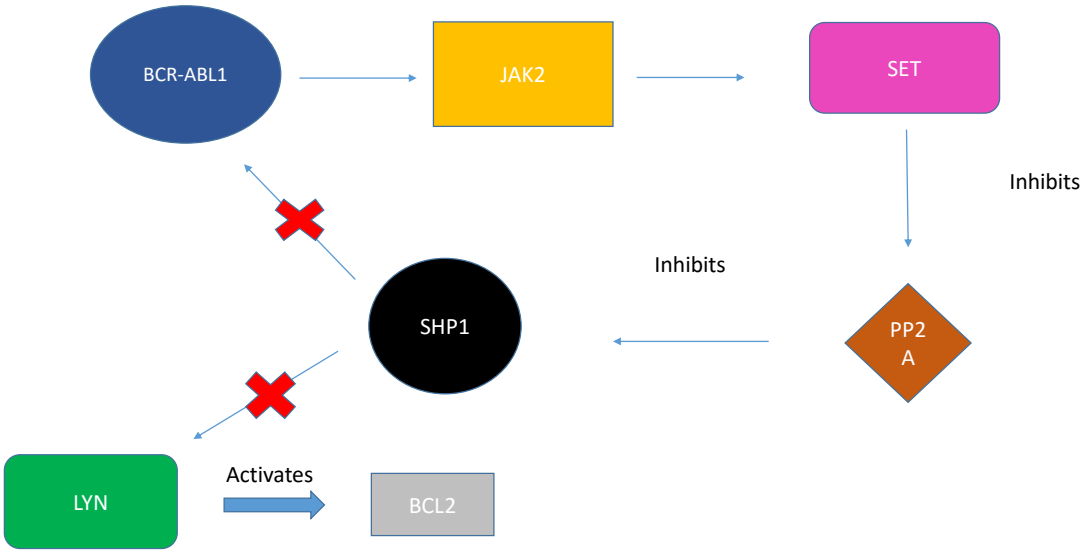


Fig. 1.15.1. Jak 2 signalling pathway. This scheme sums how the inactivation of PP2A by SET leads to the activation of cell survival signals pathways, in this case Lyn pathway. The red crosses indicate that SHP1 is no longer able to dephosphorylates BCR-ABL and to block Lyn signalling cascade (adapted from Neviani P, *et al*., 2013).

1.16. STAT 3 and STAT 5 pathways

STAT 5 and p53 have been indicated as factors that can sensitize apoptosis, through activation by Oncostatin, which is a cytokine (Chipoy C. *et al*., 2006). Pifithrin-μ has been demonstrated to inhibit p53, as mentioned previously, therefore this pathway could possibly be inhibited by Pifithrin-μ. Bortezomib also appeared to inhibit STAT5 on BCR-ABL cells and on $CD34^{+}$cells (Bucur O. *et al*, 2013). STAT3 has been targeted as one of the main signalling pathways responsible for the cellular development of CML; therefore, it has been studied to understand resistance mechanisms to some of the therapeutic options. Hoelbl A. *et al* (2010) have demonstrated that STAT 5 is incredibly important not only for the initiation process, but also for the maintenance of the disease. They used lymphocytic leukaemia cell lines to determine if STAT3 and STAT5 were involved in the resistance mechanisms; after stimulation by IFN-β, they were able to state that STAT5 and not STAT3 is able to induce resistance (Hoelbl A. *et al*, 2010). In vivo, these results are confirmed by several studies. Mice were injected with a BCR-ABL variant, the P210; the STAT5 wild-type murine hematopoietic cells resulted in a high percentage of survival, suggesting an essential role of STAT5 in the resistance to therapies (Ye D., *et al*., 2006). STAT5 seems to be also independent by JAK2; Warsch W. *et al*. (2011) tested levels of STAT5 after 24 hours treatment with Imatinib. The cells were already expressing high levels of STAT5, but after one day of treatment STAT5 increased from 20% to 56%. The experiment was repeated to JAK2 deficient cells, showing very similar results. Thus, it may indicate that JAK2 and

STAT5 could be activated independently from each other (Warsch W. *et al*, 2011). Several experiments have confirmed that the inhibition of STAT5 pathway induced apoptosis in a leukemic cell line, K562. It has been demonstrated either by treating K562 cells with a tyrosine kinase inhibitor, PD180970 which also targeted other molecular pathways (Huang *et al*, 2002). Moreover, the specific STAT5 inhibitor Pimozide has reached the cell cycle arrest and the subsequential apoptosis by targeting the genes expressing STAT5; again, the experiment was performed on K562 cell line and on KU812, another CML cell line (Nelson E.A. *et al*, 2011).

Jak2 could also be linked with STAT3 pathway, as Jak2\STAT3 is considered one of the most important pathways that lead to cell proliferation (Coppo P. *et al*, 2006 and Samanta, A. K., *et al*, 2010). Understanding its role in the resistance to therapies has become an important target from several studies. Particularly, a STAT3 inhibitor combined with Imatinib has shown that this combination is a promising therapy option; Eiring *et al* demonstrated that STAT3 has a critical role in the reduction of resistant CML stem cells to Tyrosine Kinase Inhibitors which were Imatinib and Dasatinib in this case (Eiring A.M. *et al*, 2015). As other STATs proteins, STAT3 are activated by growth factors, cytokines that gradually lead to cell survival or proliferation. Also, similarly to other STATs, JAK2 assists the phosphorylation of the C domain at the Tyr705; following, STAT3 translocates to the nucleus where it will bind a target DNA sequence. STAT3 is highly regulated by some protein-tyrosine phosphatase (PTP), which dephosphorylates Jak2. Moreover, suppressors of cytokine signaling (SOCS) and protein inhibitor of activated STATs (PIAS) are also involved. SOCS are responsible of binding Jak2 and addressing it to cell degradation, whilst PIAS can block STAT3 to bind to DNA (Nair, R. R., *et al*., 2012). Fig.1.16.1. illustrates the pathway mentioned above.

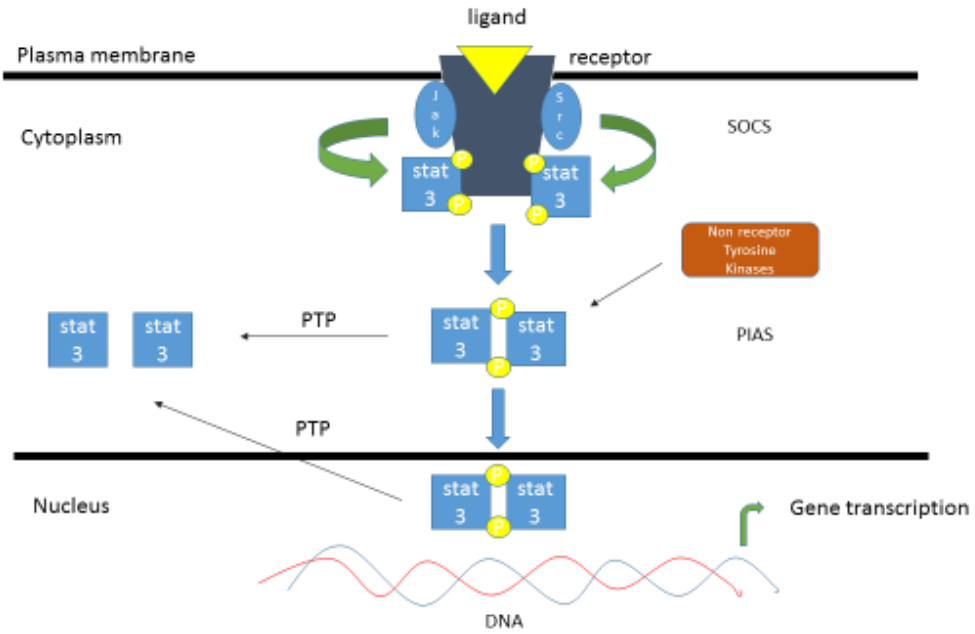


Fig 1.16.1. STAT3 pathway scheme. This figure shows the several steps of the STAT3 pathway, from the ligand binding to the regulation of the activation by SOCS, PTP and PIAS and the STAT3 targeting a gene (adjusted from Nair, R. R*., et al*, 2012 and Yu, H., *et al*., 2009).

1.17. Ras/Raf/MEK/ERK pathway

The Ras/Raf/MEK/ERK signalling pathway is also considered as a trigger cascade for the development of cell survival; one of the transcription factors to be activated at the end of the pathway is NFκB, which is inhibited by Bortezomib and HSPIs. Ras is a GTP-binding protein responsible of various kinases and the pathway mentioned before is incredibly common in many human cancers (Chang F. *et al*, 2003). It has been largely studied for its involving in the proliferation of hematopoietic cells; in fact, Raf1 is required for the growth together with other factors such as IL14, which stimulates the Ras kinase activity and IL6 (Muszynski, K. W., *et al*., 1995). It is also interesting to analyse its importance in childhood acute leukaemia, where Ras mutations pathway are implicated in the diagnosis. It is still not clear whether Ras mutations are an initiating or secondary event with respect of the disease development, however it is certain that these mutations have a high incidence in very aggressive cases of Acute Lymphocytic Leukaemia (ALL). Further studies are currently going on to understand their involving in the ALL (Knight, T., & Irving, J. A. E., 2014). Regarding the actual cascade of events, it is significant to determine the sequence of the passages that triggers this pathway, also known as Mitogen Activated Protein Kinases/Extra-signal Regulated Kinase (MAPK/ERK) pathway. Following a growth stimulus on the Epidermal Growth Factor Receptor (EGFR), the tyrosine activity of the receptor is activated; this causes the binding of GRB2 to the tyrosine residues of the receptor. SOS, a guanine nucleoside exchange factor binds to GRB2, inducing the GTP-binding protein Ras to lose a phosphate transforming GTP in GDP; this allows Raf (MAPKKK) to be activated, which subsequently activates Mek (MAPKK), which then will activate Erk (MAPK). In case of cancer and, thus leukaemia, ERK is constitutively active (Dhillon, A. S., *et al*., 2007). This pathway takes place in the cytoplasm; after the MAPK activation, a transcription factor such as c-Myc, Ets, CREB and NF-κB will be stimulated in the nucleus. Fig. 1.17.1. sums the pathway described above.

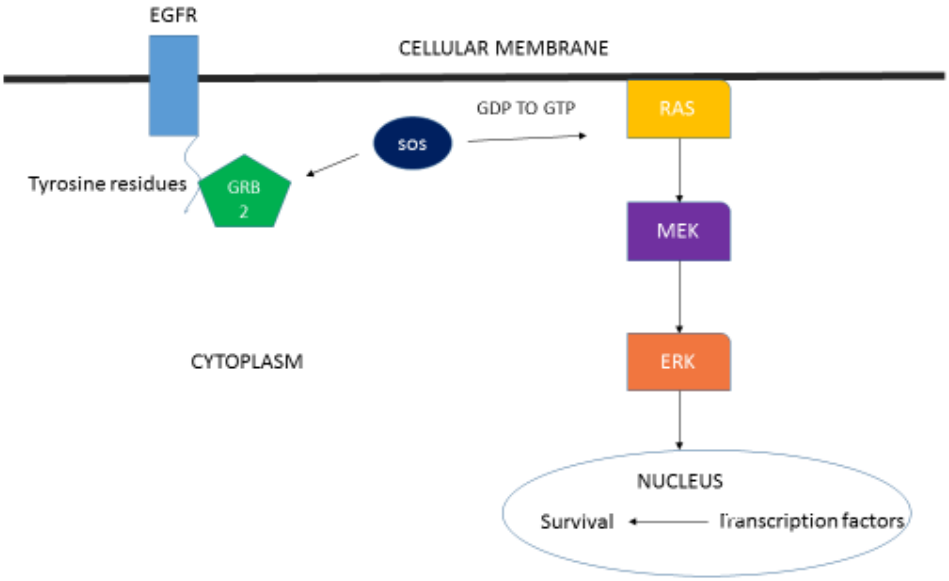


Fig. 1.17.1. The Ras protein cascade of signals. GTP binds to RAS protein that activates MEK which subsequently activates ERK protein. As a final result of the protein cascade there is the transcription of genes which stimulate cell survival (adapted from Dhillon, A. S., *et al.*, 2007 and Vojtek, A. B., & Der, C. J., 1998).

After the activation of one the transcription factors, the outcome of the cascade could follow different ways. In fact, active ERK could suppress the genes which code for the inhibition of apoptosis. When the activation is sustained, some genes are upregulated; therefore, the continuous activity goes throughout the G1 phase. Consequently, another set of genes is downregulated by ERK; the decreased expression of these genes, which are antiproliferative, allows the G1 phase to enter to the S phase and, thus, to a constant cell proliferation (Yamamoto, T., *et al.*, 2006).

1.18. PI3K\AKT pathway

Between the cell signalling pathways, PI3K\AKT is one of the most important and notable ones. Bortezomib appeared to downregulate phosphor-Akt on hepatocarcinoma cells, leading to apoptosis (Chen, K. F., *et al.*, 2011) and to leukaemia, also overregulating p53 (Bastian L. *et al*, 2013). Similar to the previous pathways mentioned, PI3K\AKT plays an important role not only in the development of the disease but also in the choice of therapeutic options. PI3K has a strong connection with RAS; following a cytokine signal, RAS is activated and binds to the PI3 110 subunit. Therefore, PI3 is activated and it translates the passage between PI2 (phosphatidylinositol 4, 5 bisphosphate) to PI3 (phosphatidylinositol 3-phosphate), as shown on Fig.1.18.1. Consequentially, AKT and its signalling cascade could take place. Akt remains inactive in the cytosol prior the PIP3 binding through PDK1; PIP3 and Akt have in fact high affinity. (Chang F. *et al*, 2003 and Duronio, V., *et al.*, 1998). Akt, which is a serine-threonine kinase, acts against apoptosis and it is high in chemotherapy and radiotherapy resistant patients (Hirai H. *et al*, 2010). Due to its clinical importance, it has become a therapeutic

target. PI3 and Akt inhibitors such as quercetin and Wortmannin have been largely tested in vitro and in vivo, showing promising apoptosis induction results in leukemic patients combined or as a single agent (Russo, M., *et al*., 2010 Marley, S. B., *et al*, 2004).

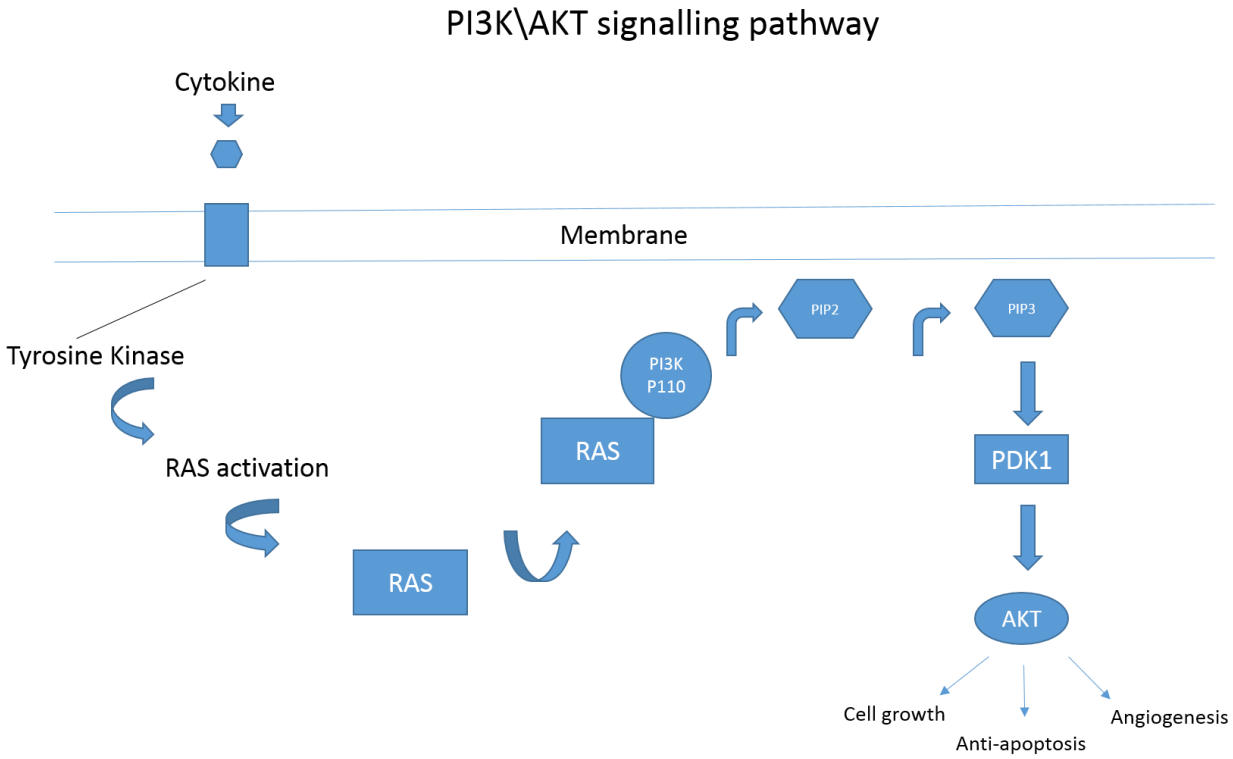


Fig.1.18.1. Overview of the PI3\AKT signalling pathway. Following an external stimuli, RAS is activated and it binds with the P110 subunit of PI3; this event triggers the addition of phosphate to PIP2 and PIP3. Through PDK1, the serine threonine Akt is then recruited, provoking the cell signalling cascades (adapted from Chang F. *et al*, 2003 and Duronio, V., *et al*., 1998).

1.19. NF-κb signalling pathway

The NF-kb (nuclear factor kappa-light-chain-enhancer of activated B cells) proteins are deeply involved in the inflammation process, as well as playing a role in cancer initiation and progression and it is inhibited by Bortezomib. (Blum, K. A., *et al*., 2007 and Roccaro, A. M., *et al*., 2006). NF-kb activation strongly depends on the release of IKK proteins or from the cleavage of factors such as p100 or p105 (Hoesel B. and Schmid J.A., 2013) Multiple stimuli can activate the IKK complex and therefore trigger the activation of NF-kb. For example, the activation of NF-kb could be a result from a stimuli which started from the Ras pathway; Ras proteins are GTP-binding protein responsible of various kinases (Schmid J.A. and Birbach A., 2008). When the GTP-binding protein Ras loses a phosphate, the consequence is the transformation from GTP to GDP; this ainduces Raf (MAPKKK) to be activated, which subsequently activates Mek (MAPKK), which then ultimately activates Erk (MAPK). This final passage is the step which stimulates the release of IKK. The signalling pathway could be canonical or alternative; the canonical pathway is triggered by factors like TNFα or IL-1 which bind to Troll-like receptors on the cytoplasm. Signalling kinases then activate the IKK complex

mentioned above, which leads to the activation of the IκB complex. Following phosphorylation activities which are paramount for the ubiquitination process, the IκB is trimmed and proteins like p50 or RelA are released in the nucleus where they will target specific genes (Zhou, J., *et al*., 2015). Instead, during the non-canonical pathway, activation of B-cell activation factor (BAFFR) receptor leads the NF-κB-inducing kinase (NIK) to activate IKKα; this phosphorylates p100 which then leads to the polyubiquitination of p100 which will result in the proteasome products p52 and RelB which subsequently can activate the transcription of target genes. In leukaemia, a NF-κb target gene like Bcl-2 has been demonstrated to be upregulated; therefore, this pathway is one of the responsible of resistance to apoptosis, whether TNF-α regulated or chemotherapy resistance apoptosis. Also, it seems that p65 and p50 NF-κB interact direct with STAT3, resulting in a direct regulation of anti-apoptotic proteins. On the other hand, it has been shown that there may be a mutual inhibition between p53 and NF-κB; the NF-κB subunit RelA has been shown to inhibit p53 activation, while p53 can irreversibly inhibit NF-κB transcriptional activity (Perkins N.D., 2007). How the mutual inhibition works is still unclear and needs further studies. The two NF-κb pathways are described in Fig.1.19.1.

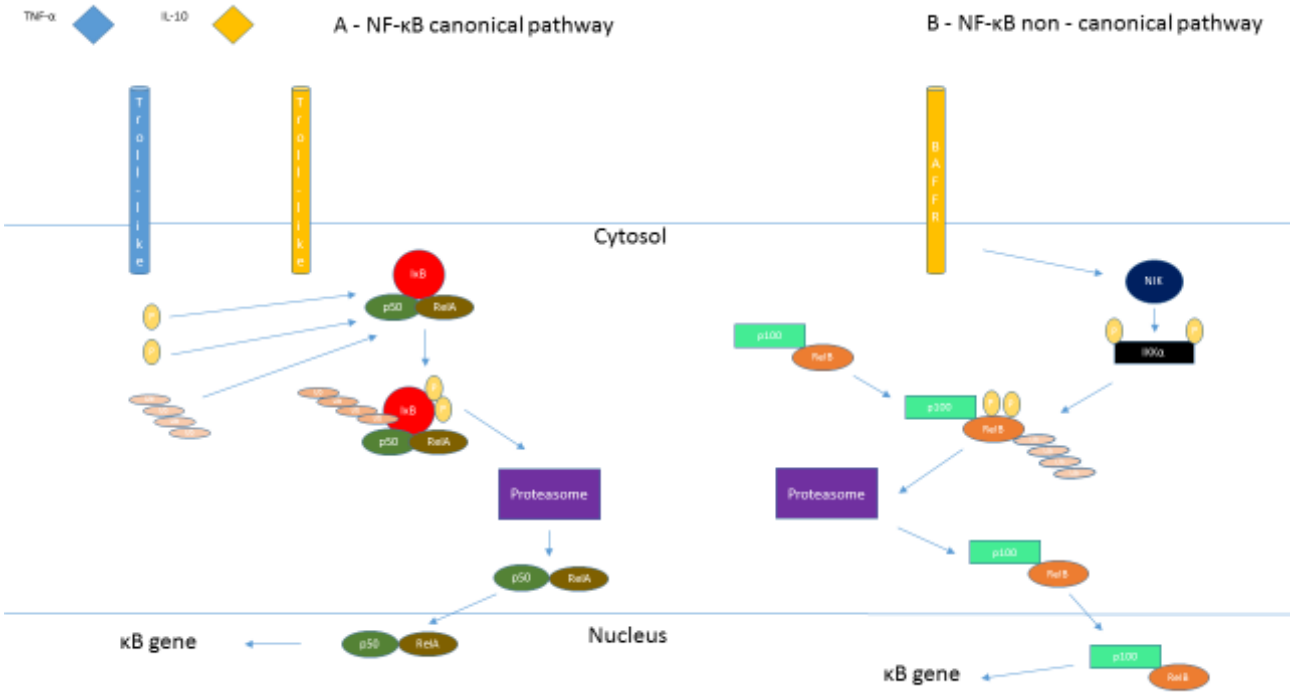


Fig. 1.19.1. Nf- kb pathway: canonical and not canonical. A: **Canonical Pathway**. Following stimulation from the final products of Ras pathway, TNF-α or IL-10 binds to Troll-like receptor which stimulate the activity of IκB complex. The ubiquitinated complex enters the proteasome, which degrades IκB and leaves p50 and RelA to transfer to the nucleus; there, they will target the Kb gene. B: not – canonical pathway. BAFFR stimulates NIK to activate IKKα; therefore p100 is phosphorylated causing polyubiquitination and activation of the κB gene (adapted from Zhou, J., *et al*, 2015 and Roccaro, A. M., *et al*., 2006).

1.20. Tumour protein 53 (p53)

This protein is deeply involved in the apoptosis and anti-apoptosis mechanisms; also, one of its function is to start the process of DNA repair following cell stress or DNA damage. Its role in cell death and\or survival is therefore paramount. P53 and the proteasome complex are strongly linked; indeed, in normal cells, p53 is downregulated by a ubiquitin ligase, MDM2 which binds to p53, causing the passage from nucleus to the cytosol where it will be recognized by the proteasome and will be thus degraded. When mutated, of course, p53 is responsible of cell survival due to the reduced or suppressed apoptosis (Wang, C., & Youle, R. J,2009). This protein therefore plays a pivotal role in cell viability by regulating apoptosis; several studies have confirmed its involvement in the mitochondrial pathway that then leads to cell survival. It seems that following stress signalling, p53 binds with members of Bcl-2 proteins in the mitochondria which will then lead to the activation of the caspase signalling cascade and, therefore, to apoptosis. More in details, the proteins belonging to Bcl-2 family can have an anti-apoptotic role or a pro-apoptotic role; pro-apoptosis proteins such as Bax, PUMA and Bak are involved in the process. Upon signalling, p53 from the nucleus induces the expression of PUMA, which will then promote the release of p53 into the cytoplasm; p53 was inactivated by binding to the anti-apoptotic protein Bcl-xL (Vaseva, A. V., & Moll, U. M., 2009). The release in the cytosol of p53, which therefore enters the mitochondria, lets the pro-apoptosis protein Bax to translocate from cytosol to mitochondria. P53, then oligomerizes Bax and Bak and inhibits the anti-apoptotic members of Bcl-2 present in the mitochondria. As a result, the event that occurs is called mitochondrial outer membrane permeabilization (MOMP), caused by p53 binding to cyclophylin D. This complex induces the release of cytochrome C from mitochondria, which subsequently starts the caspase signalling cascade that leads to apoptosis, finally. Interestingly, the endoplasmatic reticulum (ER) interplays with mitochondria during the apoptosis process. A severe ER stress induces an incremented efflux of Ca^{2+} from ER via IRE1 and PERK membrane proteins into the mitochondria; this induces the subsequent release of cytochrome C and the start of caspase cascade signalling (Bravo-Sagua R. *et al*, 2013). Importantly, Bcl-2 and Bcl-xl seem to inhibit calcium release, whereas pro-apoptotic proteins like Bax and Bak positively regulate the communication between ER and mitochondria (White C. *et al*, 2005). This proposed scheme is yet to be clear, considering that some studies suggest that the mitochondrial pathway and, broadly p53 involvement in it, is only an amplifier of the caspases cascade and not the initiator of the apoptosis process (Marchenko N.D., *et al*., 2007). Fig. 1.20.1 describes what has been mentioned above.

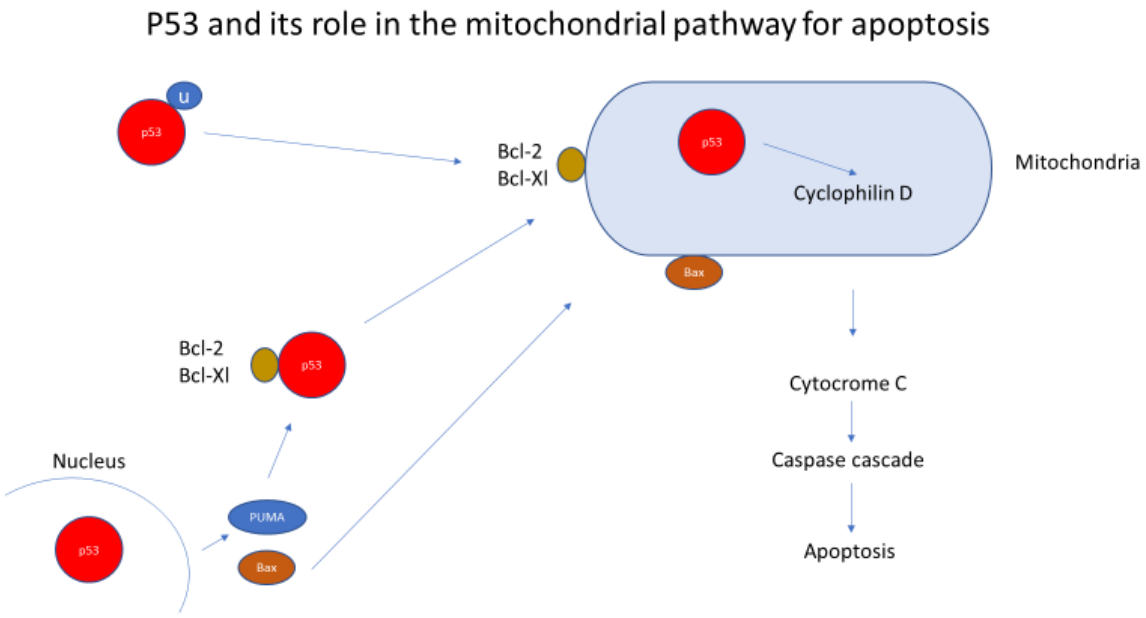


Fig. 1.20.1. The role of p53 in the mitochondrial pathway for apoptosis. p53 in the nucleus induces PUMA expression, which in turn releases p53 in the cytosol which was inactive because bound to Bcl-2 or Bcl-XL. Consequently, Bax is transported to the mitochondria, together with p53. This results in a signalling cascade which starts from Cyclophilin D, which causes the release from the mitochondria of apoptotic factors which involve cytochrome C. Then, the caspase cascade is initiated and the apoptosis takes place (adapted from Marchenko N.D., *et al*., 2007 and Vaseva, A. V., & Moll, U. M., 2009).

1.21. Aims of the thesis

A growing body of evidence demonstrated the over expression of HSPs in different type of tumours; it has also been demonstrated that HSPs could lead to cell survival, inhibition of apoptosis and cell growth in leukaemia and on other type of tumours. The importance of proteasome activity on cancer is always furthermore documented and recently it became a target of treatment on leukaemia. This led to the development of HSPIs and PIs; considering the assisting role of chaperone to the proteasome, it would be interesting to investigate how these two families of drugs could potentially enhance each other activity and affect cell viability of leukemic cells. It would also be interesting to evaluate their effectiveness in respect of apoptosis in a short time of treatment and on the lowest possible doses. Some CML and AML patients may develop resistance to conventional chemotherapy; a potential new therapy option such as a combination between HSPIs and PI could help overcoming the resistance or could potentially be considered as afirst therapy option. Furthermore, the role of HSP72 in apoptosis has not been fully investigate on leukaemia; therefore, it would be interesting to acquire better knowledge on how HSP72 could inhibited and how could this inhibition lead to apoptosis on leukemic cells. Also, the targeted therapy era has incredibly improved the specificity of the drugs and reduced the toxicity that unfortunately was common with normal chemotherapy. Therefore, it is always more relevant to understand how drugs could selectively attack only tumour cells. Often, targeted therapies can be performed with a combination of agents, aiming to improve each other mechanism of action and ultimately selectively kill cancer cells. This thesis aims to investigate effectiveness of HSPIs and Bortezomib as single agents and in a combined therapy, to further understand the role of HSP72 in apoptosis and to indicate a therapy option for CML and AML that could potentially be used in future on patients.

This will be accomplished by:

- Analyse the effects on cell viability and on apoptosis of HSPIs and Bortezomib as single agents on K562 and U937 cells.
- Analyse the effects on cell viability and on apoptosis of HSPIs and Bortezomib as combined therapy on K562 and U937 cells.
- Investigate the role of HSP72 inhibition following single agent and combination treatment of HSPIs and Bortezomib on K562 and U937 cells.
- Investigate the role of HSP72 inhibition caused by single agent and combination treatment of HSPIs and Bortezomib in apoptosis by measuring Bcl-2 levels on U937 cells.

CHAPTER 2: METHODS

2.1. BUFFERS AND SOLUTIONS

2.1.1 Cell culture sterile 10 % Antibiotic RPMI media

Cells were cultured in RPMI 1640 media which was made sterile by adding 5 ml of antibiotic and 50 ml of Fetal Bovine Serum (FBS), under a sterile cabinet.

2.1.2. Cell viability solutions

PES stock solution

0.0092 grams of PES were dissolved in 10ml of DPBS. The solution was covered in foil due to its light sensitivity and stored at -20°C.

MTS solution

MTS solution was prepared by dissolving 0.042g of MTS powder in 20ml of DPBS and the pH was adjusted to 6.5 by adding 1M HCl or 1M NaOH. The solution was then made to a final volume of 21ml by adding DPBS. MTS working solution was then made by adding 1 ml pf PES solution to 20ml of MTS. MTS solution was then stored as 1ml aliquots in micro-centrifuge tubes wrapped in foil and stored at -20°C.

2.1.3. Drug preparation

Bortezomib (5mg) was purchased by Stratech as a pale yellow powder; in order to have a 1mM concentration, Bortezomib was diluted with 13.0127 ml of DMSO, aliquot and stored at – 80 °C.

Pifithrin-μ (5mg) was purchased by Sigma Aldrich; it was diluted by adding 275.923 µl of DMSO to reach a 100 mM concentration and stored at 2-8 °C.

PES-CL (10mg) was purchased by Calbiochem as a white powder and diluted with 313.36 µl of DMSO to have a 100 mM concentration. It was then stored at 2-8 °C.

2.2. METHODS

2.2.1. Cell culture

CML blast crisis K562 cell line and AML U937 cell line were cultured in RPMI media. K562 is a suspension cell line derived from a female with CML in blast crisis and they seem to most resemble lymphocyte B cells. U937 cells are also a suspension cell line derived from the pleural effusion of a patient with histiocytic lymphoma; also, U937 are monocytes. Cells were left to grow at 37°C with 5

% CO_2 in tissue culture flask 25cm^3. The cells were sub-cultured every 2-3 days. The cells were divided at a log phase concentration and sub-cultured at a 1x10^5 cells\ml. Prior to any treatment experiment, both cell lines were centrifuged for 5 minutes at 500 xg; following, fresh media was added to the pellets, allowing the cells to reach their log phase the day of the experiment. On the day of the experiment, the cells were again centrifuged for 5 minutes at 500 xg and fresh media was added, adjusting the final cells concentration, when necessary, to 1x10^6 cells/ml.

2.2.2. Cell preservation in cryostat

K562 and U937 cells were cultured until they reached a cell density of 5 x 10 ^{5}cells/ml and the cells were centrifuged for 5 minutes at 500 xg. The culture medium was discarded and the pellet was re-suspended in 1ml freeze media (10% DMSO in FBS). The pellet was gently suspended and aliquoted into labelled cryo-tubes. The cells were then incubated in the vapour phase of liquid N2 for a minimum of 2 hours after which they were stored in a designated location in the cryostat.

2.2.3. Cell culture starting

The cryotube containing 500 µl of cells was thawed into a 37°C water bath, until the cells were defrosted. Following the defrosting process, the cells were added to 9.5 ml of RPMI in a tissue culture flask 25cm^3in order to achieve 10 ml total volume.

2.2.4. Cell counting

The cells counting was performed using a haemocytometer. A 1:1 dilution of cells with trypan blue was used to count the number of viable cells. Trypan blue is a dye which is characterized by permeating the membrane of dead cells; therefore, viable and live cells will be not coloured and the dead cells will acquire the blue color.

2.3.5 Cells treatments

2.3.5.1. HSPIs and Bortezomib single agent treatment on K562 and U937 cells for MTS assay and Annexin V\PI assay

K562 and U937 cells were plated on a 96 wells plate at a concentration of 1x10^6 cells/ml. The drugs were diluted in RPMI before administration to the cells; each HSPIs started from an initial concentration of 50 µM followed by 6 other concentrations. The range of dilutions for the HSPIs was therefore composed of 50 µM, 25 µM, 12.5 µM, 6.25 µM, 3.12 µM and 1.5 µM. Similarly, the range of dilutions for Bortezomib was composed of 250 nM, 125 nM, 62.5 nM, 31.2 nM, 15.6 nM and 7.8 nM. Once the minimal concentrations were obtained in the following experiments for HSPIs and for Bortezomib, the range for HSPIs was of 50 µM, 25 µM and 12.5 µM. Bortezomib range, following the same principle, was 31.2 nM and 15.6 nM. Each plate had also untreated live cells and dead cells that were both used as controls. The plates were incubated in the CO_2 incubator for the appropriate time (24 hours or 6 hours, depending on the experiment) of treatment.

2.3.5.2. HSPIs and Bortezomib combined treatment on K562 and U937 cells for MTS assay and Annexin V\PI assay

K562 and U937 cells were plated on a 96 wells plate at a concentration of $1x10^6$ cells/ml. The drugs were diluted in RPMI before administration to the cells; for both HSPIs the dose was 12.5µM and for Bortezomib were 15.6 nM and 31.2 nM. Then, HSPIs were added to both cell lines for one hour and Bortezomib was added subsequently following the hour treatment. The cells were then incubated for 24 hours at 37 °C. Equally, Bortezomib was added to K562 cells and U937 cells for an hour and the HSPIs were added subsequently. Each MTS plate had also untreated live cells and dead cells that were both used as controls. Plates for Annexin V\PI assay had live untreated cells control, Annexin V only live cells control, PI only live cells control and Annexin\PI live cells control. The cells were incubated for 24 hours at 37 °C.

2.3.5.3. HSPIs and Bortezomib combined treatment for HSP72 flow cytometry assay and Bcl-2 flow cytometry assay

K562 and U937 cells were plated on a 96 wells plate at a concentration of $1x10^6$ cells/ml. The drugs were diluted in RPMI before administration to the cells for both HSPIs the dose was 12.5µM and for Bortezomib were 15.6 nM and 31.2 nM, which were double diluted. Then, HSPIs were added to both cell lines for one hour and Bortezomib was added subsequently following the hour treatment. The cells were then incubated for 6 hours at 37 °C. Equally, Bortezomib was added to K562 cells and U937 cells for an hour and the HSPIs were added subsequently. The cells were incubated for 6 hours at 37 °C.

2.3.6. MTS assay

The assay measures cellular viability through the reduction of a tetrazolium compound (yellow) to a soluble formazan product which is only converted by viable cells (Riss, Niles, & Minor, 2004). The assay gives an indication of cellular metabolism and changes in cellular metabolism equate to a change in absorbance. MTS is a negatively charged molecule that is impermeable to the cell membrane, thus a cell permeable electron acceptor phenazine ethosulphate (PES) is added to the MTS solution.

For MTS assay U937 cells and K562 cells were cultured in 96 well plates. To determine cell viability post treatment, 20µl of MTS working solution was added to each well containing cells cultured in the plate. The plate was then incubated at 37°C with 5% CO2 for 2 hours for following which absorbance was read at 490nm. A dead cell control and blank were always included in the assays negative controls whilst untreated cells acted as positive control. Indeed, cell viability is calculated as (mean of sample\ mean of positive control * 100).

2.3.7. Flowcytometry analysis

2.3.7.1. Annexin V\PI assay

One of the first event of apoptosis is the translocation of the membrane phosphatidylserine (PS) from the inner membrane to the external surface. Annexin V is a FITC conjugated Ca^{2+}protein with a strong affinity to PS and binds to the cells exposing it. To measure necrosis Propidium Iodide (PI) dye is used; membranes of dead and damaged are permeable to PI. Therefore, viable cells are considered Annexin V and PI negative, early apoptotic cells Annexin V positive and PI negative, late apoptosis Annexin V positive and PI positive, because they are already dead or damaged. Necrotic cells are PI positive and Annexin V negative.

Following the treatment, K562 cells and U937 cells were transferred to a 96 wells V-bottom plate and centrifuged at 500 xg for five minutes. The supernatant was discarded and the cells were washed with iced DPBS (100 µL); the plate was centrifuged at 500 g for 5 minutes. The washing procedure was repeated for two times in total; following, supernatant was discarded and the Annexin V\PI binding buffer was prepared by diluting the Annexin V binding buffer with type 1 water. Each plate had four different controls (in triplicates): unstained cells (50 µl of staining solution only), Annexin V controls (50 µl of staining solution and 2.5 µl of Annexin V staining only), PI controls (50 µl of staining solution and 2.5 µl of PI staining only) and Annexin V\PI controls (50 µl of staining solution and 2.5 µl of both staining products). The treated samples contained 50 µl of staining solution and both Annexin V and PI staining, to obviously detect the type of cell death. The plate was then left for fifteen minutes in the dark; as a last step, 200 µl of staining solutions was added and the plate was analyzed on the flow cytometer within an hour using 488 nm excitation and 525 nm emission for FITC Annexin V and 535 nm excitation and 617 nm for PI.

2.3.7.2. HSP72 assay

Following the treatment, K562 cells and U937 cells were transferred to a 96 wells V-bottom plate and centrifuged at 500 xg for five minutes. The supernatant was discarded and the cells were washed in 100 µl of DPBS; the plate was again centrifuged at 500 xg for five minutes. Subsequently, the supernatant was discarded and the pellets were cytofixed with 70 µl of cytofix\perm buffer. After 20 minutes at 4°C, 70 µl of DPBS were added to dilute the permeabilization buffer. The plate was centrifuged and the supernatant was again removed. The cells were re-suspended in 100 µL blocking buffer (5 % FBS in DPBS) and they were left for 5 minutes at room temperature. Following, the plate was centrifuged as described above and the supernatant was removed. The antibody (HSP70 FITC – Stressmarq) solution (1:50 in blocking solution) was then prepared. Each control well was re-suspended with 50 µl of blocking solution, whilst the test samples were re-suspended in 50 µl of diluted antibody solution. The plate was left for 45 minutes covered in foil at 4°C. Then, 50 µl of blocking solution were added on top of each well. The plate was, as usual, centrifuged and the supernatant was discarded. The cells were re-suspended in 100 µl DPBS; the plate was then ready to be analyzed, although it could be read at 488nm excitation and 525 nm emission within two weeks if left at 4°C and covered in tin foil.

2.3.7.3. Bcl-2 assay

Following the treatment, U937 cells were transferred to a 96 wells V-bottom plate and centrifuged at 500 g for five minutes. The supernatant was discarded and the cells were washed in 100 µl of DPBS; the plate was again centrifuged at 500 xg for five minutes. Subsequently, the supernatant was discarded and the pellets were cytofixed with 70 µl of cytofix\perm buffer. After 20 minutes at 4°C, 70 µl of DPBS were added to dilute the permeabilization buffer. The plate was centrifuged and the supernatant was again removed. The cells were re-suspended in 100 µL blocking buffer (5 % FBS in DPBS) and they were left for 5 minutes at room temperature. Following, the plate was centrifuged as described above and the supernatant was removed. The antibody (Anti-mouse\rat Bcl2 FITC Invitrogen) solution (1:50 in blocking solution) was then prepared. Each control well was re-suspended with 50 µl of blocking solution, whilst the test samples were re-suspended in 50 µl of diluted antibody solution. The plate was left for 45 minutes covered in foil at 4°C. Then, 50 µl of blocking solution were added on top of each well. The plate was, as usual, centrifuged and the supernatant was discarded. The cells were re-suspended in 100 µl DPBS; the plate was then ready to be analyzed, although it could be read at 488nm excitation and 525 nm emission within two weeks if left at 4°C and covered in tin foil.

2.3.8. Combination index

The data of combination experiments were evaluated in order to measure antagonism, additive or synergy effects. The Compusyn software (Combosyn, Inc) was used to calculate the combination index (CI) for each combination of the drugs; particularly, the median values for each single treatment were interpolated with the median values of each combined treatment, in every experiment. The results were given by Chou-Talau equation, where CI < 1 = synergy, CI=O additive, CI > 1= antagonism.

2.3.9. Statistical analysis

All data was analysed using GraphPad Prism™ 6 version 6.05 (GraphPad Software, Inc, San Diego, USA). Importantly, all the MTS data were checked for normality prior to analysis with ANOVA. All data are presented as mean ± SD and were analyzed using either a one-way ANOVA Dunnett's post hoc test, where all the groups (drug concentrations) where compared to a control group (live cells). One-way ANOVA Tukey's post hoc test was also used to compare different drug concentrations to each other.

CHAPTER 3: HEAT SHOCK PROTEIN INHIBITORS AND PROTEASOME INHIBITOR ADMINISTERED AS SINGLE AGENTS ON K562 AND U937 CELLS AND DETERMINATION OF TYPE OF CELL DEATH

3.1. INTRODUCTION

Targeted therapy could be considered as one of the main therapeutic options against both CML and AML. In the last decade the need to reduce the side effects of chemotherapy drugs became more urgent, in an attempt to improve the life conditions of patients. Chemotherapy drugs are extremely toxic for cancer cells, but such drugs attack indistinctly normal cells also. The targeted therapy applies the same principle and has the same aim: to kill cancer cells. The main difference with classic and canonical chemotherapy relies in the specificity of the target of these new drugs. Indeed, each new biological drug belonging to targeted therapy group has a specific target and it aims to only inhibit or bind to its target (Baudino T.A., 2015). On Chronic Myeloid Leukaemia (CML) perhaps the most used of the targeted therapy drugs is Imatinib and the descendants of this drug such as Dasatinib or Ponatinib as described on section 1.6. With respect to Acute Myeloid Leukaemia (AML), recently a new tyrosine kinase inhibitor called Midostaurin was tested, giving encouraging results *in vitro*; however, it also showed that it bound to plasma proteins causing a variable and steady-state free drug concentration (Perl A.E., 2017). Interestingly, Heat Shock Protein Inhibitors (HSPIs) such as Pifithrin-μ and PES-CL are not clinically relevant currently, perhaps due to the poor knowledge on their pharmacology. It is paramount therefore to try to investigate more their mechanism of action and the effects on apoptosis, which is what this study proposes to do. Similarly, Bortezomib is not considered as one of the main options for the treatment of CML and AML; it is in fact currently used for treatment of multiple myeloma mainly. It also has been tested on other type of cancer such as lung cancer and colorectal cancer (Taromi S., *et al*, 2017 and Mañas A. *et al*., 2017). Further studies are needed to understand its role in the treatment and management of CML and AML. This study may be one of the first to indicate these two families of drugs as a novel new therapy for CML and AML.

HSPIs were designed to selectively bind to HSPs, according to their specific molecular weight. Due to their specificity, HSPIs directly bind to their chaperone target and they disrupt their resistance activity to apoptosis, leading them to cell death (Guo F. *et al*, 2005). Among specific HSP70 inhibitors there are Pifithrin-μ and PES-CL, described on section 1.15. The drugs have been shown to have an important anticancer activity on different cell lines, including leukemic cell lines such as K562, U937 and HL-60 (Kaiser M. *et al*., 2011). They seem to specifically bind with HSP70 inhibiting its protein folding activity. Consequently, it leads to cell cycle arrest and induces apoptosis in AML cell lines, in a dose-dependent manner. However, it seems to be not similarly effective in respect of CML cell lines and primary cells; this has been found following the administration of Pifithrin-μ as single agent for a long period of time (72 hours) on different cell lines, including leukemic cells (Kaiser M. *et al*., 2011). PES-CL has been developed to improve Pifithrin-μ effectiveness. It has been demonstrated that it targets and leads to inactivation of several HSP90 and HSP70 target proteins, similarly to PFT-μ (Balaburski G. *et al*, 2013). On this research study, PES-CL seemed to be more efficient with respect of apoptosis induction when compared to other three HSPIs; also, it is interesting to note that the PES-CL dose which induced a loss of cell viability and induction to apoptosis is less high than the IC_{50} found on the other HSPIs (Budina-Kolomets *et al*., 2014).

Bortezomib is perhaps the most important, well known and most used of the proteasome inhibitors. Bortezomib occupies the space where a damaged or an unwanted protein enters the proteasome; consequently, the proteins cannot enter the complex and the resulting cell stress leads to cell death by apoptosis (Hideshima T. *et al*., 2011). On leukaemia, increasing concentrations of Bortezomib (4 nM, 8 nM, 16 nM) could induce apoptosis to CML cell lines (Ba/F3 and mutated BCR\ABL cells) after 48 and 72 hours incubation. Caspase 3-assay and viable cell counts revealed the effectiveness of Bortezomib (Heaney N.B. *et al*, 2010). Also, flow cytometry analysis indicated that 50 nM of Bortezomib induced apoptosis following 48 hours on four different AML cell lines such as, HEL, KG-1, MV4-11 and HL-60 (Colado E. *et al*, 2008). Bortezomib has also been recently tested on relapsed patient with ALL, giving encouraging results and suggesting it as a new potential therapeutic option (Bostrom B., 2016). The activities of the HSP and the proteasome are strictly related; due to its role as a chaperone, HSPs assist the proteasome in recruiting the proteins that need to be folded and it helps the folding process. Particularly, HSP72 seems to be responsible of avoiding the protein aggregation and promote appropriate folding (Esser, C. *et al*., 2004). Bortezomib targets different pathways; however, the main one seems to be the nuclear factor-kappa B (NF-κB) pathway. This transcription factor is constitutively activated in breast tumour, colon cancer, prostate cancer, lymphoid malignancies and leukaemia. It therefore became target a research and clinical target; Bortezomib seems to inhibit the ubiquitination process of IKK complex, selectively binding to the proteasome. This stops the transcription of NF-κB gene in the nucleus, avoiding cell proliferation or cell survival (Hsu, S. *et al*., 2015). Bortezomib seems to induce apoptosis to multiple myeloma cells when it is added to multiple myeloma cells as single agent upregulating pro-apoptosis protein such as Noxa and disrupting cl-1 activity (Gomez-Bougie P. *et al*., 2007). Interestingly, Bortezomib induces apoptosis to CML cells like K562 and LAMA 84 following 24 hours or 48 hours treatment at low dose (4.8 nM); this could have a significant impact on CML treatment, considering the resistance to classic treatment with tyrosine kinase inhibitors (Yu C., *et al*., 2003).

The aims of this chapter are essentially four:

- To investigate the effectiveness of HSPIs (Pifithrin-μ and PES-CL) and the proteasome inhibitor Bortezomib as single agents on K562 cells and U937 cells.
- To find a potential range of concentrations to use in a subsequent series of experiment where the drugs are used in combination.
- To investigate the type of cell death that occurs following treatment.
- To verify the effectiveness of HSPIs (Pifithrin-μ and PES-CL) and the proteasome inhibitor Bortezomib as single agents on K562 cells and U937 cells in a short time of treatment.

3.2. METHODS

3.2.1 Cell culture

CML cell line K562 and AML cell line U937 were used for these set of experiments. The cells were cultured and subdivided as described in chapter 2.3.1. Both cell lines were treated at a $1x10^6$ cells\ml concentration to test drug effectiveness at the maximum logarithmic cell growth.

3.2.2. Drug dilutions

The drugs used on this chapter are two HSPIs (Pifithrin-μ and PES-CL) and a proteasome inhibitor (Bortezomib). The drugs were prepared and treated as described in Chapter 2.3.5.1.

3.2.3. MTS assay

MTS assay was performed as described on Chapter 2.3.6 following 24 hours of treatment with the drugs mentioned on section 3.2.2., in order to investigate the effects on cell viability. Subsequently, cell viability was tested following a shorter time of treatment of 6 hours. This was because it seemed interesting to evaluate the effects of the drugs within 24 hours. The choice of 6 hours was arbitrary.

3.2.4. Flow cytometry

Following the different treatments, the cells were prepared and analysed as described on Chapter 2.3.7.1. – Annexin V\PI assay.

3.2.5. Statistical analysis

All the statistical analysis were performed according to what described on Chapter 2.3.9. The IC_{50} for each drug has been calculated by calculating a non-linear fit curve.

3.3. RESULTS

3.3.1. Heat shock protein Pifithrin-μ as single agent and its effect on metabolic activity of K562 and U937 cell lines following 24 hours administration

K562 cells, at a concentration of $1x10^6$ cells\ml, were administered with Pifithrin-μ and then incubated for 24 hours. Cell viability was analysed through MTS assay, as mentioned on section 3.2.3. The initial range of concentration of Pifithrin-μ started from 1.5 μM to 50 μM. The first concentration, 50 μM, effectively resulted in compromised cell viability on K562 cells; only 4.79 % ($P<0.01$) of the cells resulted still viable. A close result was obtained also at 25 μM, where the viability was reduced to 6.32 % ($P<0.01$). 12.5 μM was very effective also, although the effectiveness of the drug does not reach what showed on 25 μM and 50 μM; 18% ($P<0.01$) of K562 cells were still viable. Then, 6.2 μM administration caused to 44.5 % ($P<0.001$) of the cells to lose their viability. At 3.125 and 1.5 μM, the metabolism of the cells was not sufficiently compromised; respectively, 77.2 % ($P<0.01$) and 74.3 % of the cells were still alive following the administration of Pifithrin-μ. Also, 50 μM, 25 μM and 12.5 μM were not significantly different when compared to each other. (Fig. 3.3.1.1.).

U937 cells, at a $1x10^6$ cells\ml concentration were administered with Pifithrin-μ and then incubated for 24 hours. Cell viability was analysed through MTS assay, as briefly described above. Again, the initial range of concentration of Pifithrin-μ that was tested was from 1.5 μM to 50 μM. 50 μM caused almost a total compromised cell viability on U937 cells; particularly, 18.29 % ($P<0.0001$) of U937 cells survived to the treatment. 25 μM and 12.5 μM showed also an almost equal percentage of cells with intact cell viability. Respectively, 41.29 % ($P<0.0001$) and 40.58 % ($P<0.01$) were the percentage with respect of these two concentrations. At 6.2 μM, 3.125 μM and 1.5 μM, the viability was not sufficiently compromised; at 6.2 μM it resulted as 88.51 %, at 3.125 μM the cell viability was detected as 85.05 % and at 1.5 μM, 87.04 % ($P<0.01$) of U937 cells still maintained cell viability. In conclusion, the top three concentrations were also significantly different from the live control, confirming a strong effect of Pifithrin-μ. Also, 50 μM, 25 μM and 12.5 μM were not significantly different when compared to each other. (Fig. 3.3.1.2.).

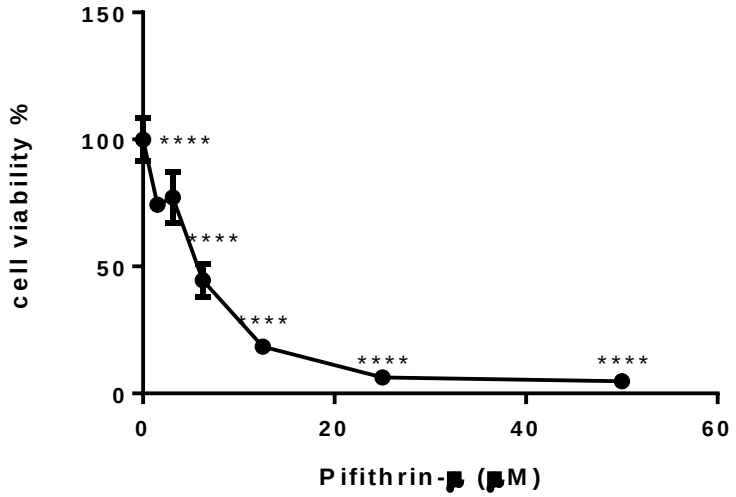


Figure 3.3.1.1. Effects on K562 cells (1x10^6 cells\ml) cell viability after 24 h treatment with Pifithrin-μ. After 24 h incubation, MTS assay was performed. Data are presented as mean ± SD, n=4. **** (P<0.0001) using one-way ANOVA Dunnett's post hoc test. Data are all compared to live control and were normalized with dead cells control (not plotted). **IC_{50}: 5.83 µM.**

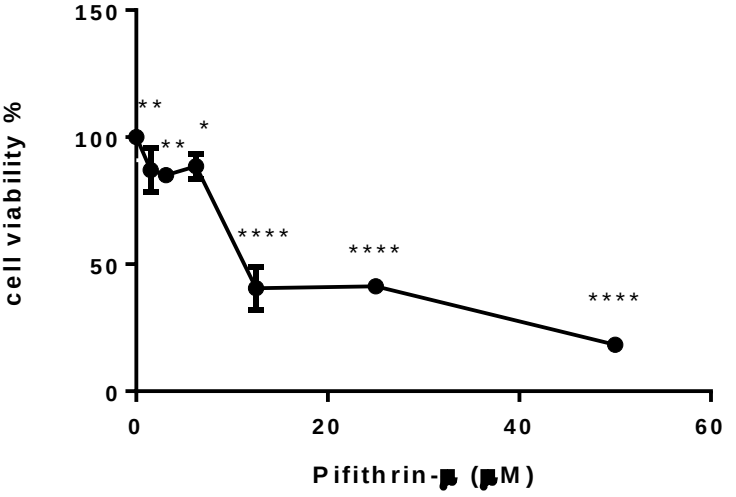


Fig 3.3.1.2. Effects on U937 cells (1x10^6 cells\ml) cell viability after 24 h treatment with Pifithrin-μ. After 24 h incubation, MTS assay was performed in order to measure cell viability. Data are presented as mean ± SD, n=4. * (P<0.05), ** (P<0.01), **** (P<0.0001) using one-way ANOVA Dunnett's post hoc test. Data are all compared to live control were normalized with dead cells control (not plotted). **IC_{50}: 9.89 µM.**

3.3.2. Heat shock protein Pifithrin-μ administered as single agent for up to six hours on K562 cells and subsequent determination of kind of cell death on flow cytometry

Following the determination of effectiveness of Pifithrin-μ after 24 hours treatment, it was interesting to understand the drug effects on cell viability in a shorter time of administration; also, it seemed important to detect what kind of cell death (apoptosis or necrosis) occurred following Pifithrin-μ. Therefore, K562 and U937 cells were treated with Pifithrin-μ up to 6 hours; notably, every hour the cells were administered with Pifithrin-μ until the sixth hour. Then, the metabolic activity was assessed through MTS assay. The drug concentrations chosen for this experiment were 12.5 μM, 25 μM and 50 μM, which are the concentrations that showed to surely affect the cell viability to more than 50 % of both K562 and U937 cell lines.

On K562, at 12.5 μM and following six hours of Pifithrin-μ administration, 49.12 % ($P<0.0001$) of the cells resulted in having a compromised cell viability. Similarly, following five and four hours of treatment the effect of Pifithrin-μ on cell metabolic activity, resulting in respectively 47.68 % ($P<0.01$) and 50.99 % ($P<0.001$) of cells with normal viability. Three hours following Pifithrin-μ administration, the percentage of K562 cells with normal metabolic activity increased to 64.11 % ($P<0.01$). Consistently, two hours of administration caused less effects on cell viability, resulting in 69.09 % ($P<0.05$) of still viable cells. After 1 hour, there is still an effect; indeed, 75.39 % of cells resulted to have a normal cell viability, indicating that a short time of administration could still cause damage to leukemic cells. Throughout the time course, there are some statistical differences; all the hours are significantly different from the 0 hours control, confirming the cell effectiveness at any time of administration (Fig. 3.3.2.1. A).

At 25 μM, 6 hours of administration affected K562 cells cell viability; more than half of the cells resulted in damaged cell metabolic activity. Indeed, 36.86 % ($P<0.0001$) of cells survived to Pifithrin-μ treatment. Five hours of administration gave a similar effect, which resulted in 38.40 % ($P<0.01$) of still viable cells. Four hours of administration still caused an effect on cell viability, 46.92 % ($P<0.001$) of cells did not have an affected metabolic activity. Following three hours of treatment, half of cells, 52.96 % ($P<0.01$), were still viable. The last two hours of administration left 58.15 % ($P<0.05$) of K562 cells still with not affected cell viability; a close result was given following an hour treatment, which resulted in 60.03 % ($P<0.05$) live cells. Given these results, 6 hours of administration resulted in a very significant statistic difference compared to control; generally, there was a significative difference throughout the entire time course (Fig. 3.3.2.1 B).

The last concentration, 50 μM, resulted in a better response on K562 cells. Indeed, following six hours of treatment, 43.95 % ($P<0.01$) of K562 cells were not affected by the treatment. Five hours of administration caused a similar effect, only 45.49 % of cells were still live. An almost equal result was found following four hours and three hours of administration; respectively, 53.68 % ($P<0.01$) and 54.69 % ($P<0.05$) of K562 cells were not affected by Pifithrin-μ. Consistently, after two hours of administration the cells that were still alive were slightly more than half, 57.51 % ($P<0.05$). One hour of administration at 50 μM still have a relevant effect on K562 cells; 42.17 % ($P<0.0001$) of the cells were still viable (Fig. 3.3.2.1. C).

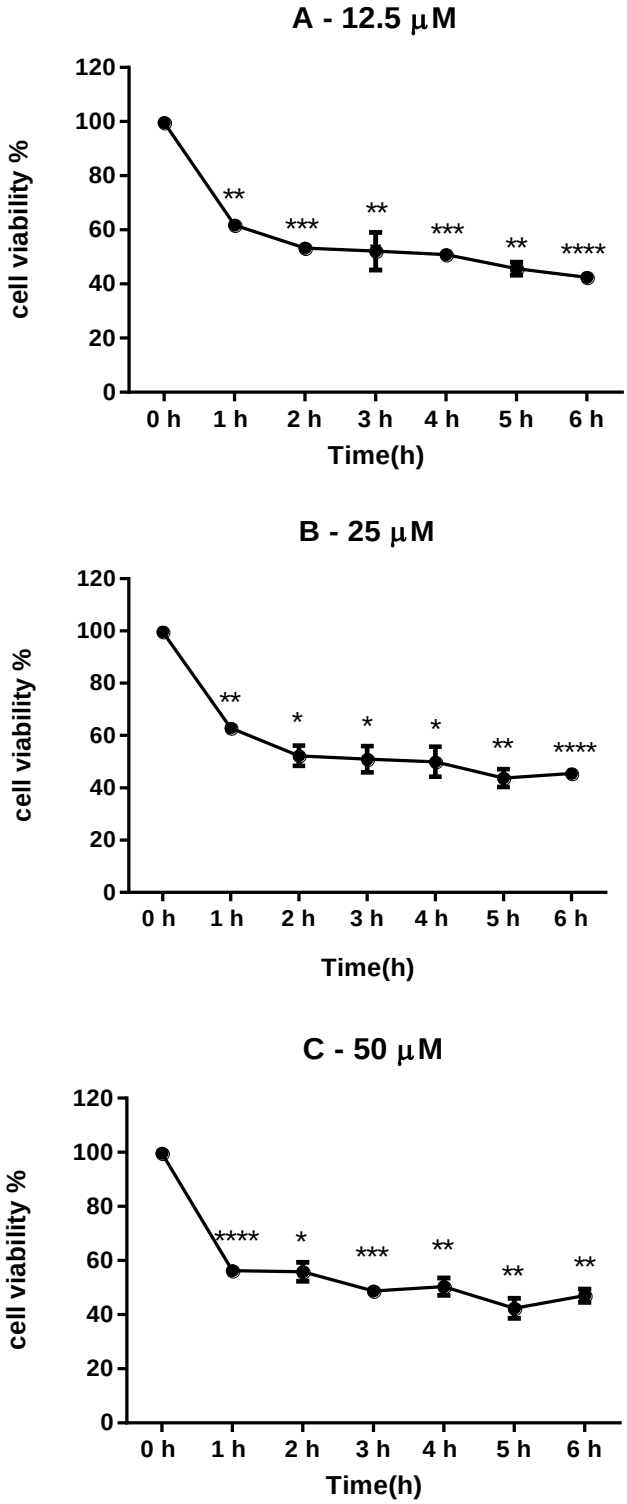


Fig 3.3.2.1. Pifithrin-μ administration up to 6 h followed by MTS assay on K562 (1x10^6). MTS solution was administered to cells after 6 hours of treatment; there is no significant difference between 12.5 μM (A), 25 μM (B) and 50 μM (C). The drug starts to be effective after 1-2 hours at all three concentrations. Data are presented as mean ± SD *(P<0.05), ** (P<0.01), *** (P<0.001), **** (P<0.0001) using one-way ANOVA Dunnett's post hoc test. Data are all compared to 0 h control and were normalized with dead cells control (not plotted).

Consequently, a confirmation of how the cells were dying was expected. In order to detect the type of cell death, Annexin V\PI assays were performed on the flow cytometer. The cells were plated and treated under the same conditions and concentrations of the MTS assays, therefore over a period of up 6 hours Pifithrin-μ was administered to K562 cell lines. Considering that on the MTS assays, the time course between 4 hours and 2 hours did not show any significant difference, the drugs were administered at 1 hours, 3 hours and then after 6 hours. The plate was then prepared for flow cytometry analysis as described in the methods section. Annexin V\PI experiment strongly suggested that the cause of death is apoptosis, confirming what MTS results partially had shown. No necrosis has been detected throughout the entire time course, at any concentration. On K562 cells, 12.5 μM is sufficient to induce early apoptosis to 35.3 % of the cells following just 1 hour of treatment. Also, 7.4 % of the cells died by late apoptosis. An equal result is visible at 25 μM, where again 35.3 % of K562 cells died by early apoptosis; equally, 7.3 % of the cells died by late apoptosis. Following 1 hour of treatment 50 μM induced 34.9 % of early apoptosis, whilst 6.5 % of the cells died by late apoptosis (Fig. 3.3.2.2. Graph B, C, D respectively). Following 3 hours of treatment, 12.5 μM induced early apoptosis to 35.6 % of cells and 6.8 % was detected as late apoptotic. 25 μM showed a similar result, indeed 30.3 % of the cells died by early apoptosis and 7.9 % by late apoptosis. 50 μM increased the percentage of early apoptosis to 46.6 % of cells and to 10.8 % of late apoptosis (Fig. 3.3.2.2. Graph E, F, G, respectively). 6 hours after the administration of Pifithrin-μ, the percentage of cells that died by early apoptosis at 12.5 μM was of 44.4 % and 15.5 % of late apoptosis. At 25 μM, 37.4 % of cells died by early apoptosis and 9.4 % by late apoptosis. Lastly, at 50 μM 6 hours of Pifithrin-μ administration induced early apoptosis to 30.5 % and late apoptosis to 5.9 %. (Fig. 3.3.2.2. Graph H, I, J).

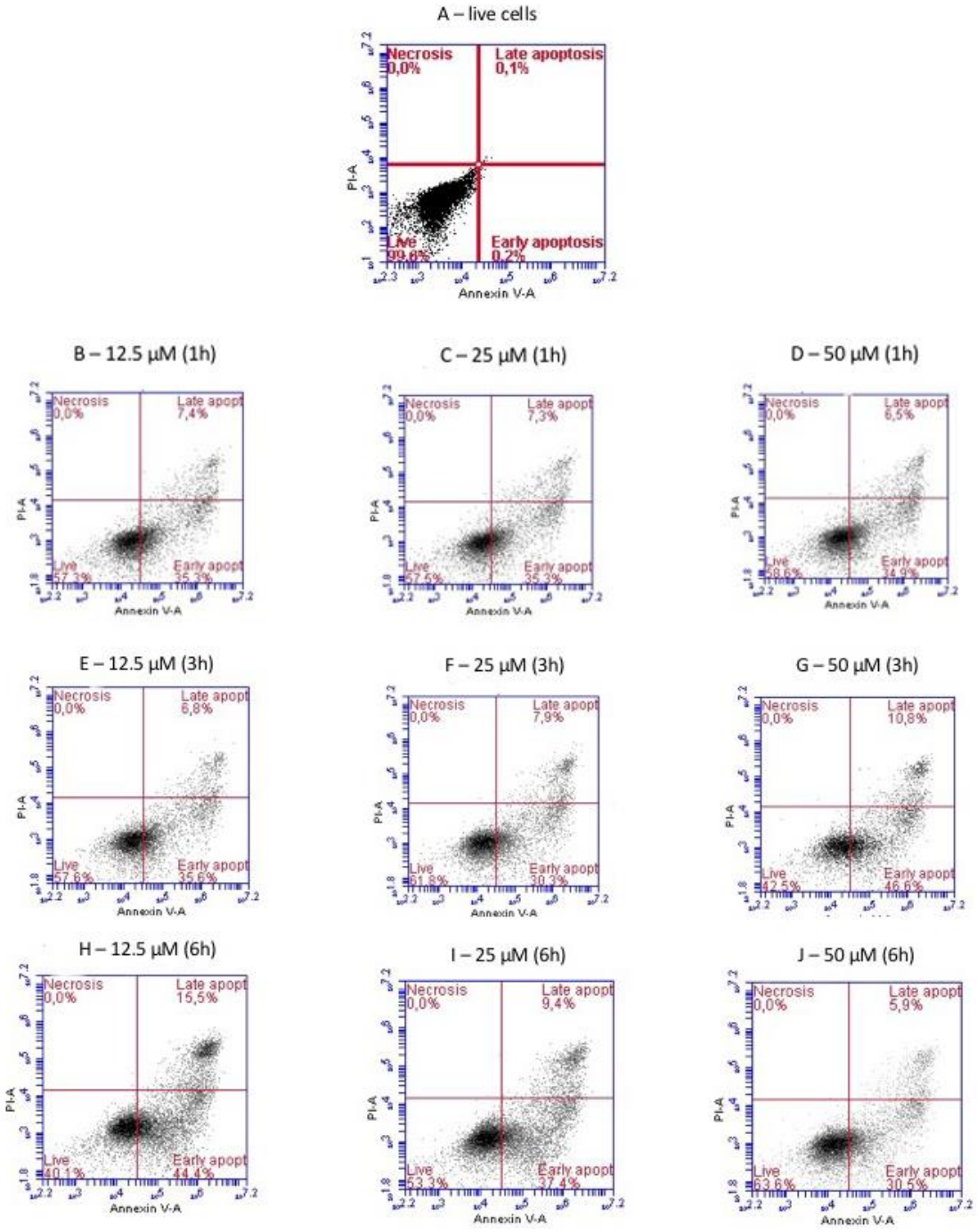


Fig 3.3.2.2. Annexin V\PI assay of Pifithrin-µ after 1, 3 and 6 hours of treatment at various concentrations on K562: the flow cytometry analysis was performed on K562 cells ($1x10^6$) following a 1, 3 and 6 hours treatment at 12.5 µM, 25 µM and 50 µM. Respectively: A (untreated live control cells), B (1 h 12.5 µM), C (1 h 25 µM), D (1 h 50 µM), E (3 h 12.5 µM), F (3 h 25 µM), G (3h 50 µM), H (6 h 12.5 µM), I (6 h 25 µM), J (6 h 50 µM). Data are representative of one replicate, as an example. Overall the samples were analysed in triplicates for each treatment and for the control.

It is also showed on Fig. 3.3.2.3, Fig. 3.3.2.4 and Fig. 3.3.2.5. The results of the statistical data of the four quadrants of the previous data: live cells, early apoptosis, late apoptosis and necrosis. For each quadrant, all the single agent concentrations were compared to live control cells at 0 h, in order to statistically evaluate the effectiveness of the treatments in terms of apoptosis. The graph shows that at 12.5 μM throughout the entire time range the apoptosis levels are different ($P<0.0001$) after 6 hours, 3 hours and 1 hour when they were all compared to live control 0 h (B). The remaining viable cells percentages are statistically significant as ($P<0.0001$) at every time of administration when compared to live cells 0 h (A). A modest percentage of the cells died by late apoptosis, at 1 hour and 3 hours the data were considered significantly different (C) from live control. Levels of necrosis are not existent (D).

The statistic display of 25 μM is shown on the following Fig. 3.3.2.4. Early apoptosis statistical data indicated that following 6 hours, 3 hours and 1 hour of treatment the difference found was $P<0.0001$, in comparison with 0 h control cells (B). Live and viable cells quadrant showed that following 1 hour and 6 hours treatment the difference with 0 h control was calculated as ($P<0.0001$) and also after 3 hours in comparison with 0 h control (A). Late apoptosis percentages are not statistically significant and no cells died by necrosis (C and D, respectively).

As shown on Fig. 3.3.2.5. 50 μM early apoptosis data were significantly different from live 0 h control as ($P<0.0001$) at every time of administration (B). With respect of live cells, viable cells percentages resulted as different from 0 h control as $P<0.0001$ throughout the entire time course, equally (A). Late apoptosis statistical data indicated that 1 hour treatment was statistically different to 0 h live cells control as $P<0.05$ and $P<0.01$ following 3 hours and 6 hours (C). As shown previously on the other concentrations, no necrosis was detected (D).

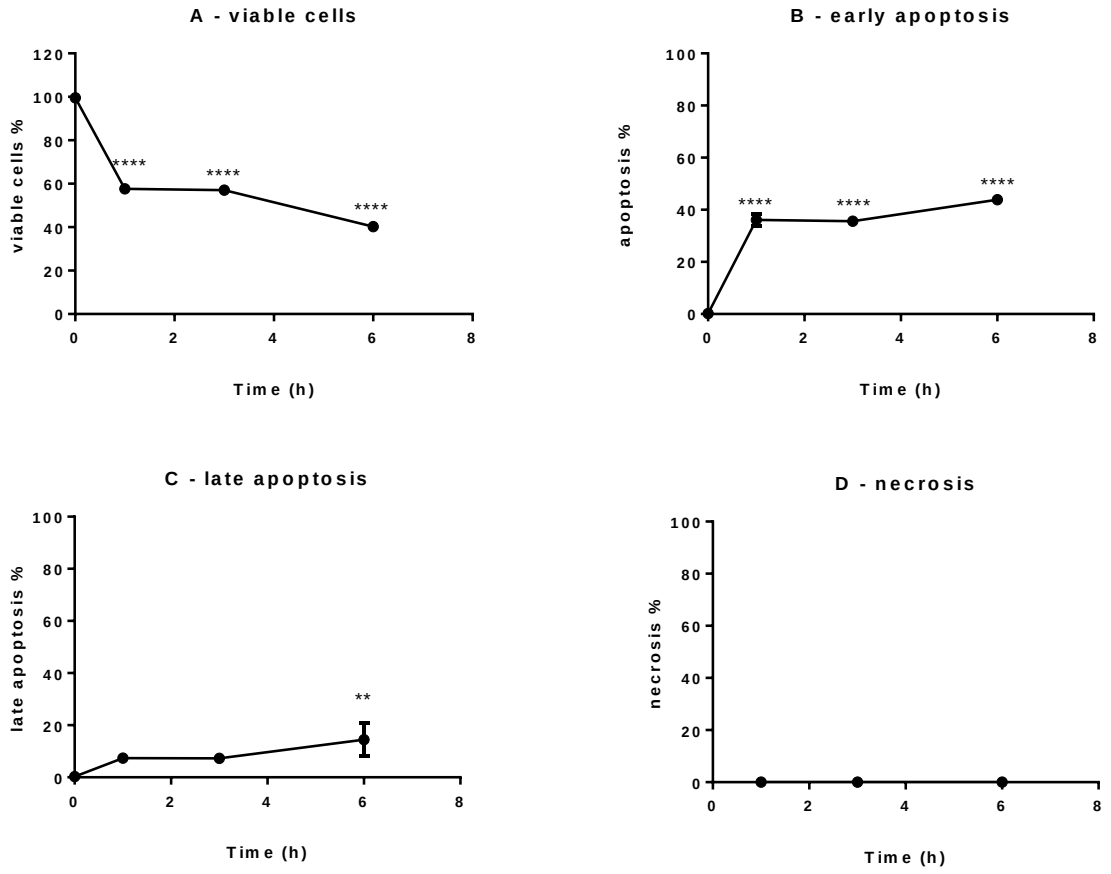


Fig. 3.3.2.3. Effects of Pifithrin-μ (12.5 μM) on K562 cells on viable cells, necrosis, late apoptosis and apoptosis after 1 h, 3 h, 6 h administration. K562 cells ($1x10^6$ cells\ml) were treated at 12.5 μM for 1 h, 3 h and 6 h and then analysed through flow cytometry to detect apoptosis and necrosis levels. A: viable cells levels, B: early apoptosis levels, C: late apoptosis, D: necrosis levels. Data are presented as mean ± SD, n=3. **** (P<0.0001) using one way ANOVA Dunnett's post hoc test. Data are all compared to live control.

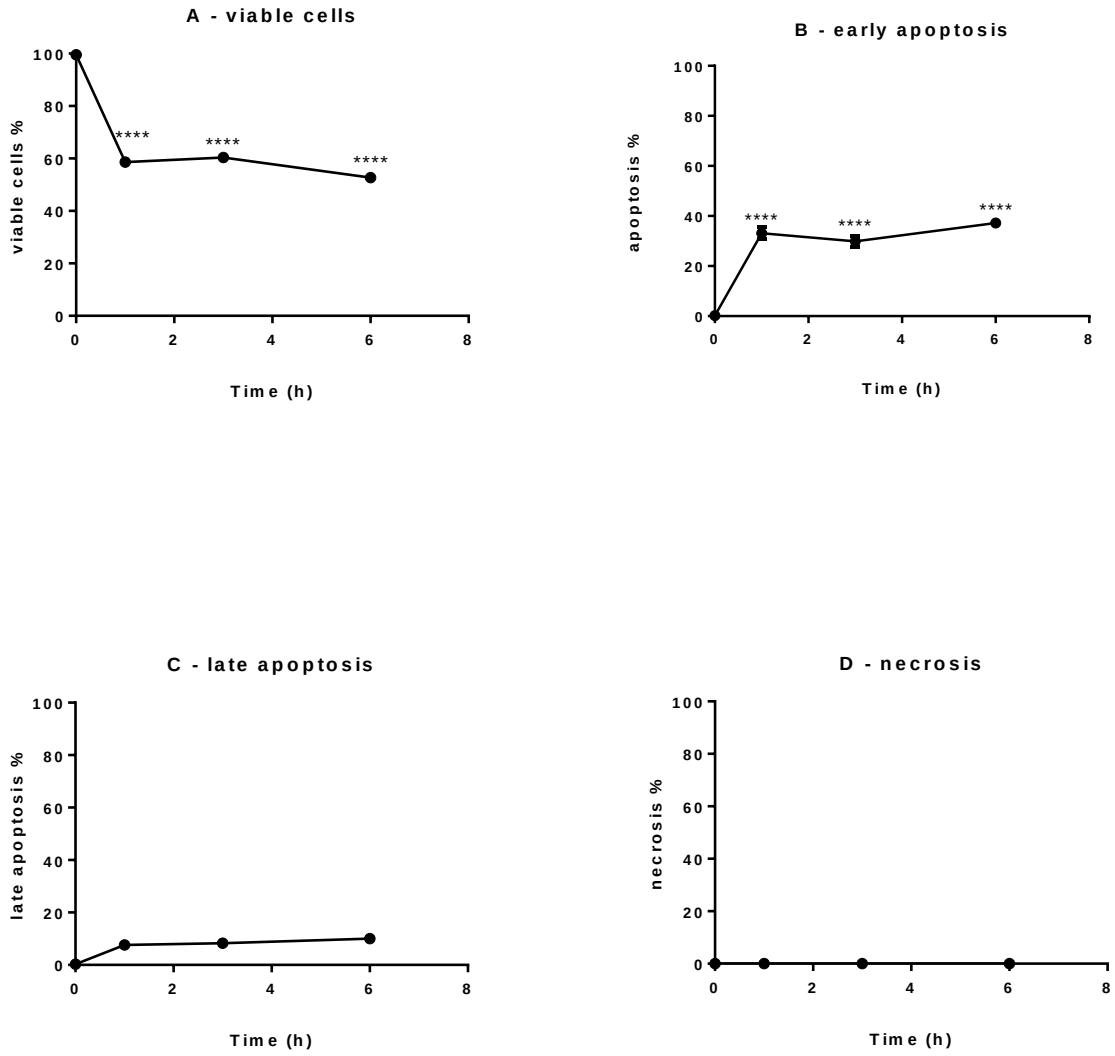


Fig. 3.3.2.4. Effects of Pifithrin-μ (25 μM) on K562 cells on viable cells, necrosis, late apoptosis and apoptosis after 1 h, 3 h, 6 h administration. The drug was administered to K562 cells ($1x10^6$ cells\ml) at 25 μM for 1 h, 3h and 6 h and its effect was then analysed through flow cytometry to detect apoptosis and necrosis levels. A: viable cells levels, B: early apoptosis levels, C: late apoptosis, D: necrosis levels. Data are presented as mean ± SD, n=3. **** ($p<0.0001$) using one way ANOVA Dunnett's post hoc test. Data are all compared to live control.

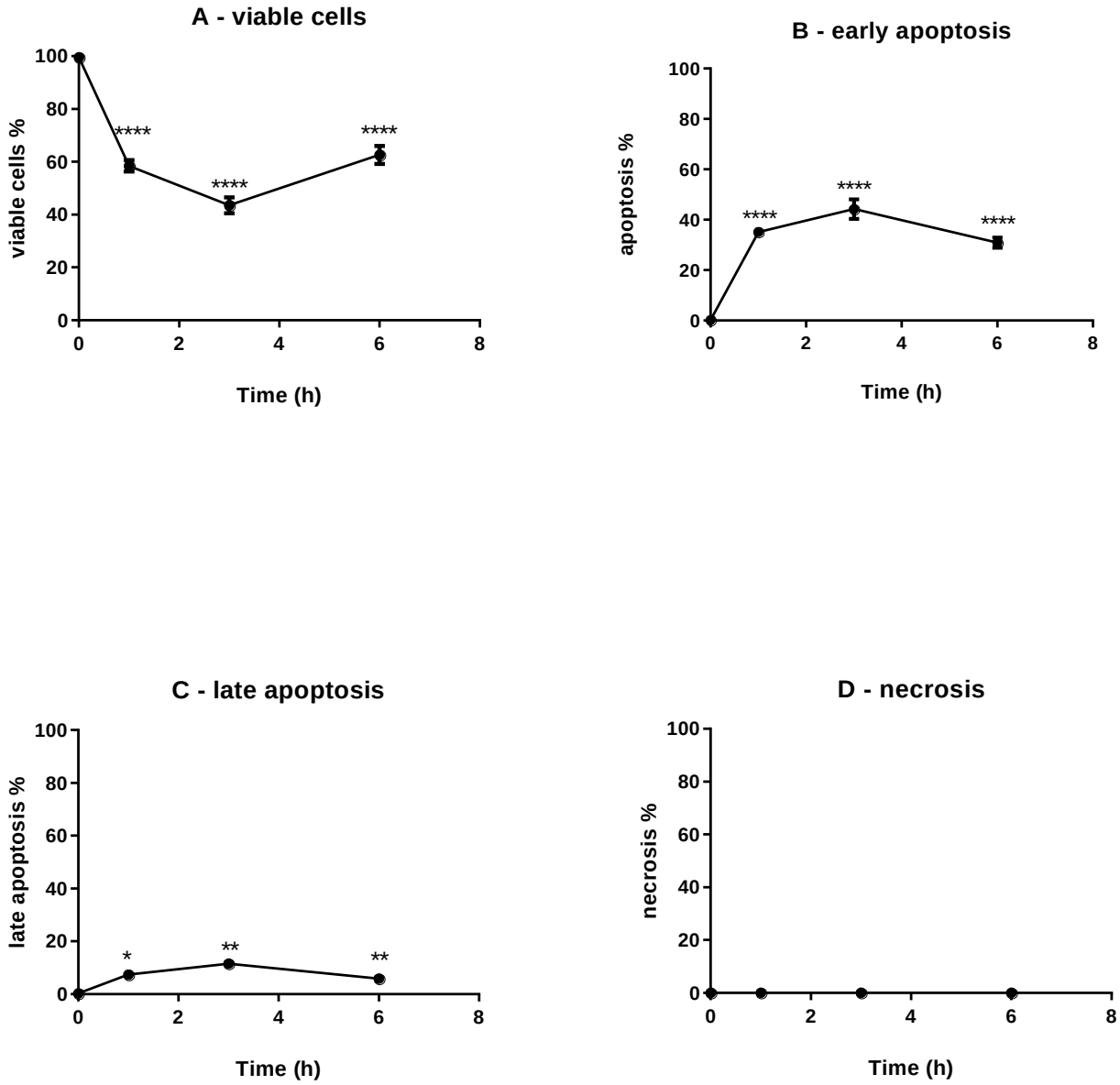


Fig. 3.3.2.5. Effects of Pifithrin-μ (50 μM) on K562 cells on viable cells, necrosis, late apoptosis and apoptosis after 1 h, 3 h, 6 h administration. K562 cells ($1x10^6$ cells\ml) were treated at 50 μM for 1 h, 3 h and 6 h and then analysed through flow cytometry to detect the type of cell death. A: viable cells levels, B: necrosis levels, C: late apoptosis, D: early apoptosis levels. Data are presented as mean ± SD, n=3. * (P<0.05), ** (P<0.01), **** (P<0.0001) using one way ANOVA Dunnett's post hoc test. Data are all compared to live control.

Moreover, it seemed interesting to further analyse statistical data of the flow cytometry quadrants compared to the time course and not to live control, as shown in the graphs above. Therefore, the comparison between one hour of treatment and both three and six hours of treatment was considered. Also, the statistical differences between three and six hours were analysed. Interestingly, there has not been a significant statistical difference between one hour and three hours in any of the cell conditions, excluding a $P<0.01$ difference for viable cells at 50 µM. Also, when statistical data of one hour treatment were compared to six hours data, the only notable differences were calculated as $P<0.0001$ on 12.5 µM and as $P<0.05$ on 25 µM on late apoptosis. Lastly, more statistical differences were found between three and six hours. $P<0.05$ was found on all the cell conditions at 50 µM and on 25 µM late apoptosis. $P<0.01$ was calculated on early apoptosis at 12.5 µM, whilst $P<0.0001$ on 12.5 µM viable cells. The remaining cell conditions were not significantly different when compared to each other (Table 3.3.2.1.)

CELL CONDITION	1 HOUR VS 3 HOURS SIGNIFICANCE	1 HOUR VS 6 HOURS SIGNIFICANCE	3 HOURS VS 6 HOURS SIGNIFICANCE
12.5 µM viable cells	Not significant	P<0.0001	P<0.0001
12.5 µM late apoptosis	Not significant	Not significant	Not significant
12.5 µM early apoptosis	Not significant	Not significant	P<0.01
25 µM viable cells	Not significant	Not significant	Not significant
25 µM late apoptosis	Not significant	P<0.1	P<0.1
25 µM early apoptosis	Not significant	Not significant	Not significant
50 µM viable cells	P<0.01	Not significant	P<0.05
50 µM late apoptosis	Not significant	Not significant	P<0.05
50 µM early apoptosis	Not significant	Not significant	P<0.05

Table 3.3.2.1. Further statistic comparing significance between the different times of administration of Pifithrin-µ on K562 cells. The table shows the statistical differences between 1 hour and 3 hours administration, 1 hour and 6 hours administration and between 3 hours and 6 hours administration. All the flow cytometry quadrants were considered, apart from necrosis which have not been detected in previous analysis.

3.3.3. Heat shock protein Pifithrin-μ administered as single agent for up to six hours on U937 cells and subsequent determination of kind of cell death on flow cytometry

At the lowest concentration, 12.5 μM, U937 cells started to show compromised cell viability after six hours of treatment; 42.45 % ($P<0.0001$) of the cells were still alive at this time of administration. Similarly, following five hours of treatment the effect of Pifithrin-μ on cell viability resulted in 45.64 % ($P<0.01$) of cells which remained alive. Also, in the period between four hours to two hours, the effect caused by the drug administration resulted in an increase of the cells with normal metabolic activity; four hours of treatment resulted in 50.87 % ($P<0.001$) cells still viable, three hours resulted in 52.12 % ($P<0.01$) and two hours resulted in 53.22 % ($P<0.001$). After 1 hour, there is still an effect, indeed 61.76 % of U937 ($P<0.01$) were not affected by the treatment (Fig. 3.3.3.1. A). At 25 μM, 6 hours of Pifithrin-μ treatment seemed to affect cell viability to slightly more than half of U937 cells; 45.45 % ($P<0.0001$) of the cells were still live after treatment. 5 hours of administration appeared to give a similar effect, where 43.47 % ($P<0.01$) were not affected by the inhibitor. Interestingly, 4 hours and 3 hours treatment were similar in their results with respect of cell viability. 4 hours of treatment resulted in 50 % ($P<0.05$) of surviving cells and 3 hours resulted in 50.93 % ($P<0.05$) of not affected cells. Pifithrin-μ continued to have a similar effect following 2 hours of treatment, 52.28 % ($P<0.05$). Also, one hour of administration at 25 μM still had an effect to U937 cells, affecting 40 % of cells; cell metabolic activity was not affected for 62.89 % ($P<0.01$) of cells (Fig. 3.3.3.1. B). The last concentration tested, 50 μM, confirmed the effectiveness found on the two previous concentrations. 47 % ($P<0.01$) of cells did not have an affected cell viability following 6 hours of administration, followed by 42.32 % ($P<0.01$) live cells after 5 hours. Similar to 25 μM, Pifithrin-μ affect metabolic activity to 50 % following 4 hours of treatment; 50.32 % cells ($P<0.01$) were not affected. A close result was observed following 3 hours of treatment, where 48.77 % of U937 cells ($P<0.001$) were still alive. The last two hours of treatment gave very similar results in respect of cell metabolic activity; 2 hours of treatment did not affect only 55.82 % of U937 ($P<0.05$), whilst one hour resulted in 56.24 % of cells not affected ($P<0.0001$), as it is shown on Graph C of Fig. 3.3.3.1. There is no significant difference in effectiveness of the drug following 6 hours of treatment throughout the range of concentrations. At the minimum dose tested, the results after 6 hours are not different from the highest one, indicating that 12.5 may be sufficient enough to affect cell viability. More importantly, Pifithrin-μ starts to be effective after 1 h, affecting cell viability of U937 cells at the concentrations tested.

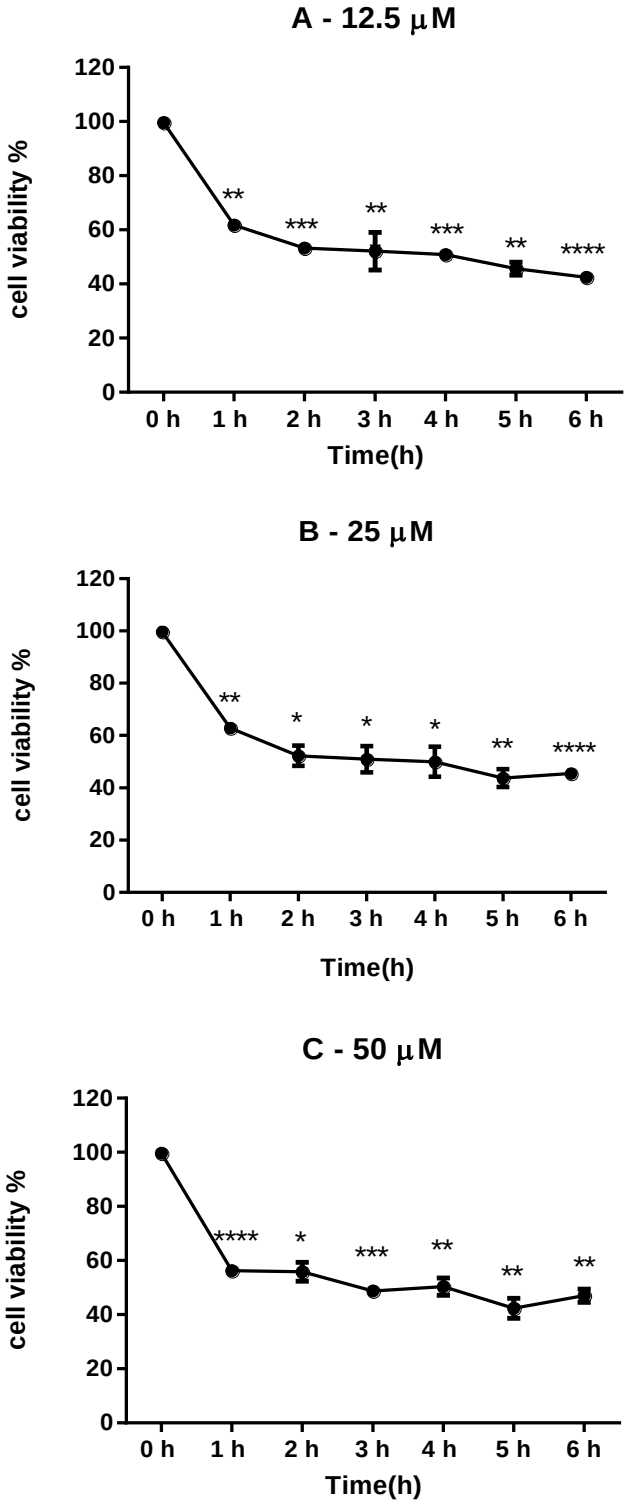


Fig 3.3.3.1. Pifithrin-μ administration up to 6 h followed by MTS assay on U937 (1x10^6). MTS solution was administered to cells after 6 hours of treatment; there is no significant difference between 12.5 μM (A), 25 μM (B) and 50 μM (C). The drug starts to be effective after 1-2 hours at all three concentrations. Data are presented as mean ± SD, n=3 * (P<0.05), ** (P<0.01), *** (P<0.001), **** (P<0.0001) using one way ANOVA Dunnett's post hoc test. Data are all compared to 0 h control and were normalized with dead cells control (not plotted).

It then became fundamental to understand how the cells die, if by apoptosis or by necrosis, in the same fashion as described on K562 cells paragraph above. Therefore, Annexin V\PI assays were performed on the flow cytometer. The same conditions and concentrations of the MTS assays were applied, thus over 6 hours both Pifithrin-μ and PES-CL were administered to both cell lines at 12.5 μM, 25 μM and 50 μM. Similar to K562 cell lines, the approach taken was to administer the drugs at 6 hours, 3 hours and then after 1 hour, considering the not significative difference between 4 hours and 2 hours. The plate was then prepared for flow cytometry analysis as described in the methods section. Apoptosis was the main cause of death, a result that is equivalent to what found on K562 cell line. Necrosis was not detected, consistent to what has been found previously.

Following one hour of treatment at 12.5 μM, 34.9 % of U937 died by early apoptosis and 6.5 % died by late apoptosis. At 25 μM, also, 35.3 % of U937 cells were detected as early apoptosis, whilst 7.3 % of U937 cells died by late apoptosis. The last concentration, 50 μM, showed equal results to 25 μM; in fact, 35.3 % of the cells died by early apoptosis, 7.4 % were detected as late apoptotic. Following 3 hours, at 12.5 μM the results were similar to what found at 1 hour of administration; indeed, 35.6 % of cells died by early apoptosis, whilst 6.8 % by late apoptosis. At the following concentration, 32.4 % of U937 cells were found early apoptotic and 9.2 % late apoptotic. At 50 μM, there was an increase to 46.6 % of cells which died by early apoptosis, followed by an increase of 10.8 % of late apoptotic cells. Finally, 6 hours of Pifithrin-μ administration resulted in 30.5 % of early apoptotic cells and 5.9 late apoptotic at 12.5 μM. When the cells received 25 μM of Pifithrin-μ, 37.4 % of them died by early apoptosis and 9.4 % of them died by late apoptosis. At the highest concentration, 50 μM, early apoptosis percentages were of 44.4 % and late apoptosis were 15.5 %, confirming the general trend that shows that 50 μM increased the induction of apoptosis on U937 cells throughout the different times of administration.

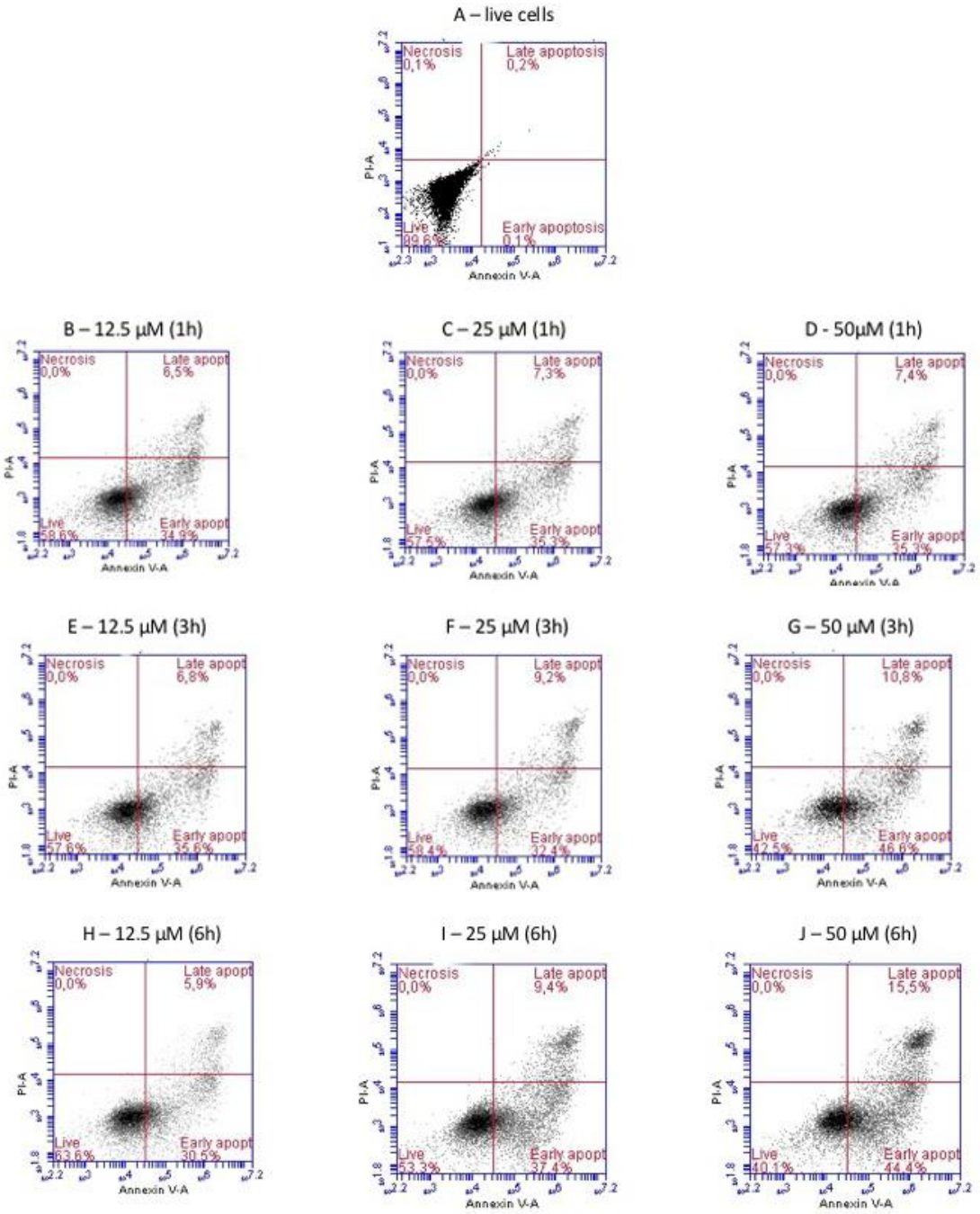


Fig 3.3.3.2. Annexin V\PI assay of Pifithrin-µ after 1, 3 and 6 hours of treatment at various concentrations on U937: the flow cytometry analysis was performed on U937 cells ($1x10^6$) following a 1, 3 and 6 hours treatment at 12.5 µM, 25 µM and 50 µM. Respectively: A (untreated live control cells), B (1 h 12.5 µM), C (1 h 25 µM), D (1 h 50 µM), E (3 h 12.5 µM), F (3 h 25 µM), G (3h 50 µM), H (6 h 12.5 µM), I (6 h 25 µM), J (6 h 50 µM). Data are representative of one replicate, as an example. Overall the samples were analysed in triplicates for each treatment and for the control.

Statistics were also plotted and examined; in the same manner of the previous section, each graph represents one of the four quadrants that are displayed in the previous pictures. For each quadrant, all the single agent concentrations were compared to live control cells, in order to statistically evaluate the effectiveness of the treatments in terms of apoptosis. With respect of 12.5 μM, Fig. 3.3.3.3. (A) showed that Pifithrin-μ is effective throughout the entire time range. The viable cells levels revealed a statistical difference of ($P<0.0001$) after 6 hours, 3 hours and 1 hour in comparison with live cells control. The early apoptotis quadrant showed that statistically significant differences, $P<0.0001$ at 1 hour, $P<0.0001$ following 3 hours and $P<0.0001$ following 6 hours of administration when they were all compared to live cells control (B). A modest percentage of the cells died by late apoptosis, at 6 hour and 3 hours the data were considered significantly different as $P<0.01$ (C) from live control. Levels of necrosis are not existent (D).

The graph representing 25 μM statistical data is shown on the following figure 3.3.3.4. Early apoptosis statistical data indicated that the values were significantly different to live cells control as ($P<0.0001$), throughout each time of administration (B). Live and viable cells data resulted different to control as $P<0.0001$ following 6 hours, $P<0.0001$ following 3 hours treatment and after 1 hour they were still significantly different as $P<0.0001$ (A). Late apoptosis percentages are again different as $P<0.01$ on each time of treatment and no cells died by necrosis (C and B, respectively).

As shown on Fig. 3.3.3.5. 50 μM induced early apoptosis data were significantly different from live control as $P<0.0001$ after each time of administration (B). The analysis of viable cells percentages resulted to show a statistical difference from control as ($P<0.0001$) following 1 hour of administration, as ($P<0.0001$) following 3 hours and $P<0.0001$ after 6 hours (A). Late apoptosis statistical data indicated that all the time of treatment gave statistical difference as $P<0.05$ in comparison to live cells control (C). Consistent to previous graphs of the other concentrations, no necrosis was detected (D).

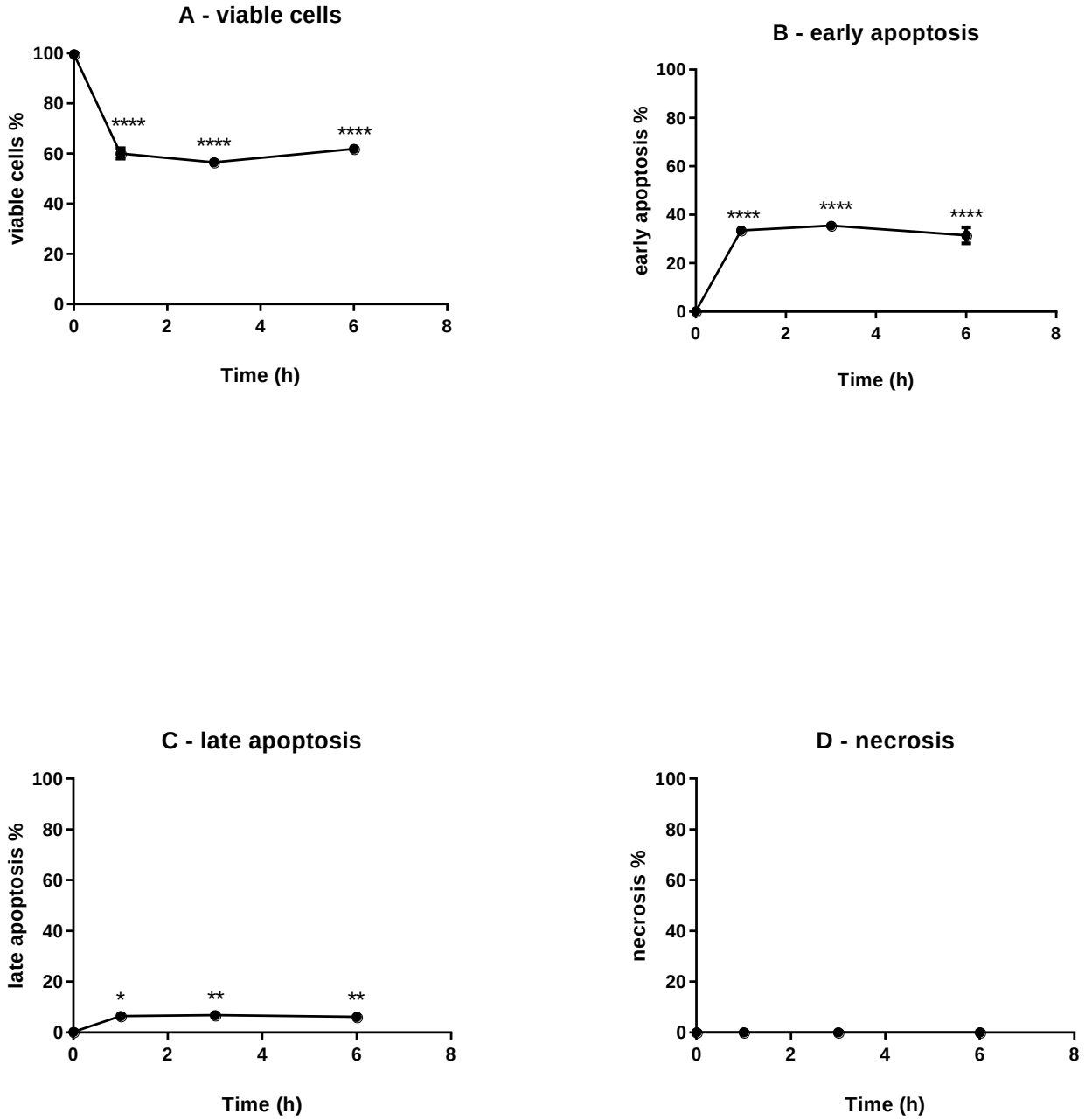


Fig. 3.3.3.3. Effects of Pifithrin-μ (12.5 μM) on U937 cells on viable cells, necrosis, late apoptosis and apoptosis after 1 h, 3 h, 6 h administration. U937 cells ($1x10^6$ cells\ml) were treated at 12.5 μM for 1 h, 3 h and 6 h and then analysed through flow cytometry to detect apoptosis and necrosis levels. A: viable cells levels, B: early apoptosis levels, C: late apoptosis levels, D: necrosis levels. Data are presented as mean ± SD, n=3. * (P<0.05), ** (P<0.01), **** (P<0.0001) using one way ANOVA Dunnett's post hoc test. Data are all compared to live control.

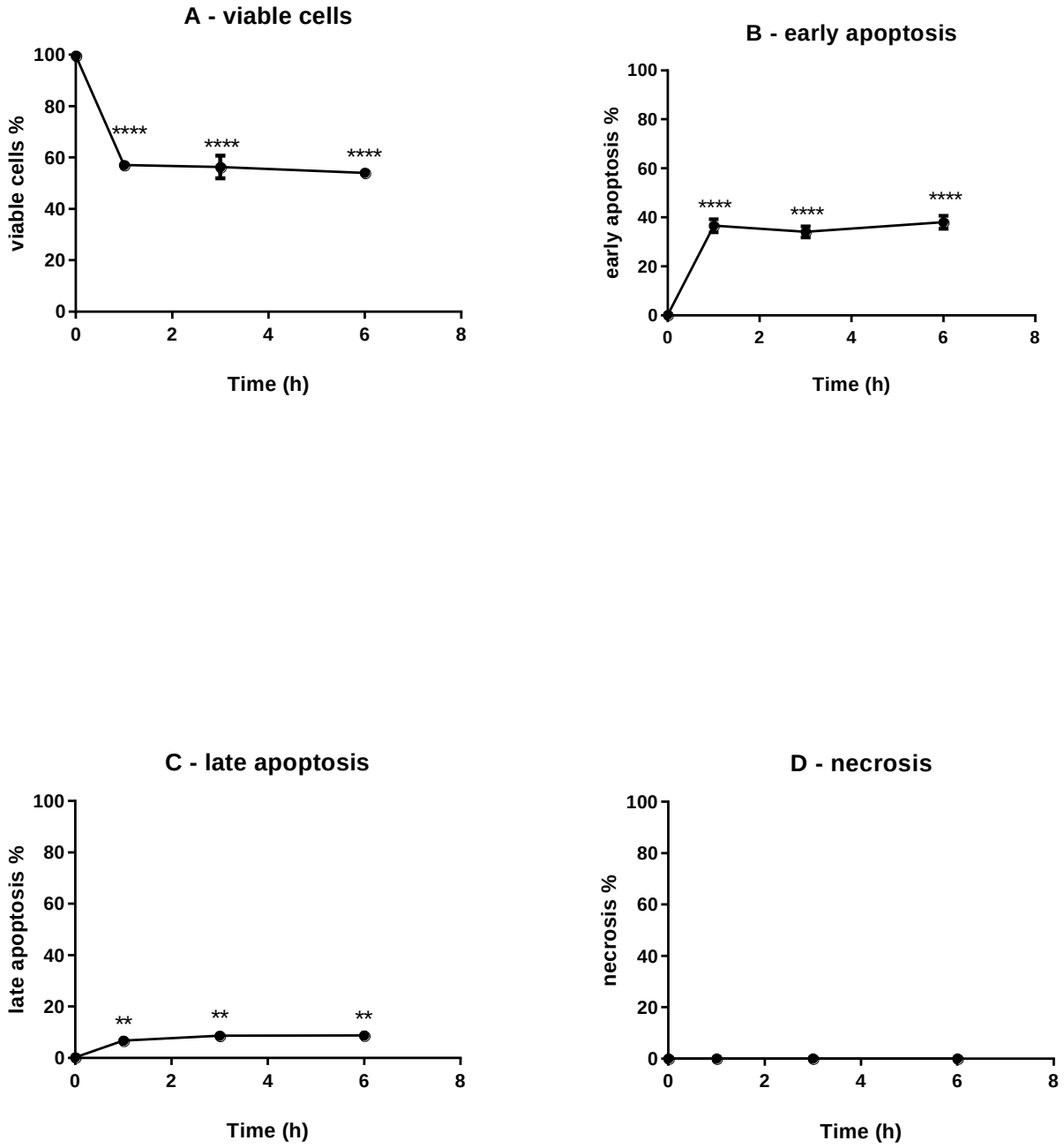


Fig. 3.3.3.4. Effects of Pifithrin-μ (25 μM) on U937 cells on viable cells, necrosis, late apoptosis and apoptosis after 1 h, 3 h, 6 h administration. U937 cells ($1x10^6$ cells\ml) were treated at 12.5 μM for 1 h, 3 h and 6 h and then analysed through flow cytometry to detect apoptosis and necrosis levels. A: viable cells levels, B: early apoptosis levels, C: late apoptosis, D: necrosis levels. Data are presented as mean ± SD, n=3. * (P<0.05), ** (P<0.01), **** (P<0.0001) using one way ANOVA Dunnett's post hoc test. Data are all compared to live control.

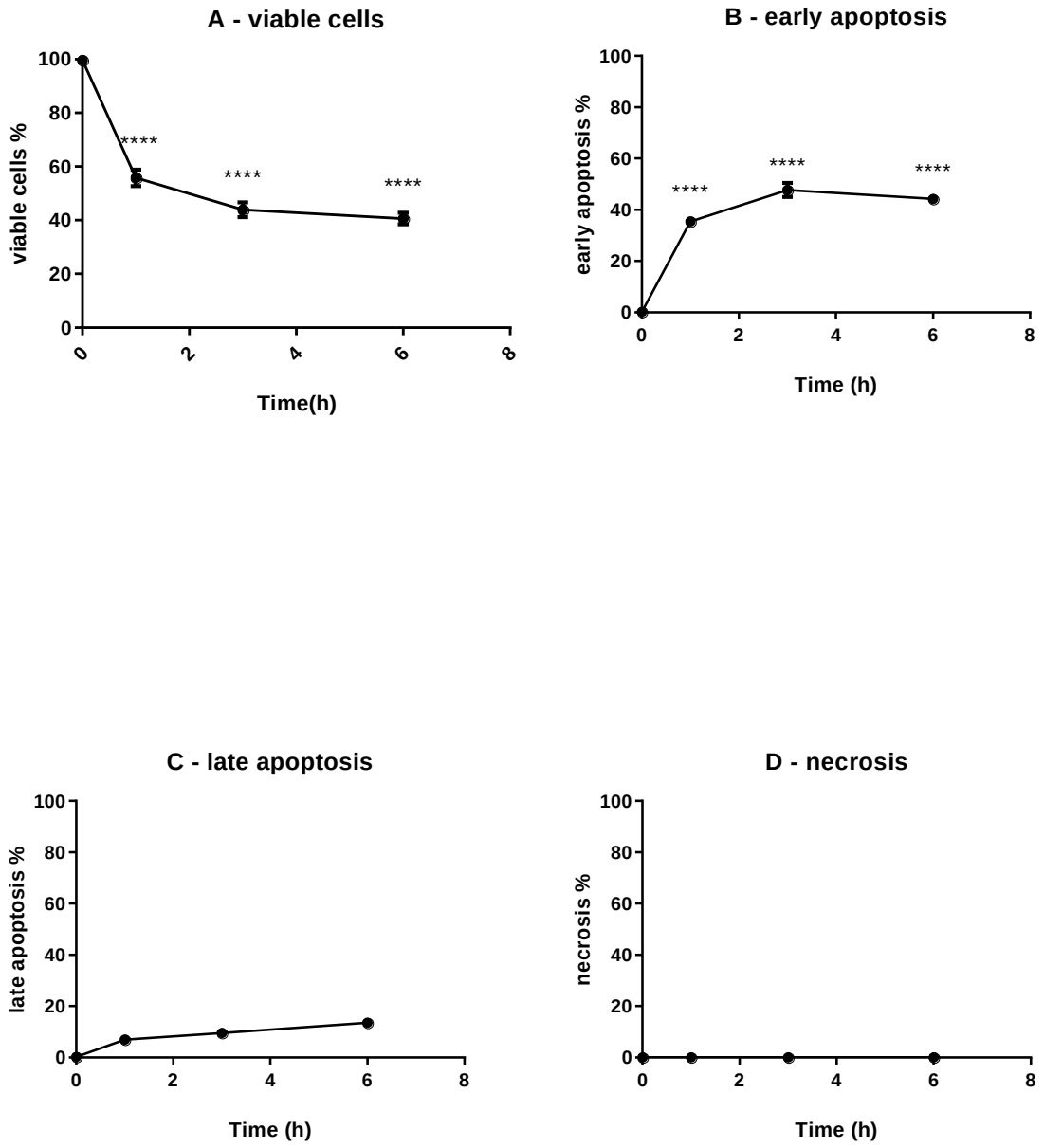


Fig. 3.3.3.5. Effects of Pifithrin-μ (50 μM) on U937 cells on viable cells, necrosis, late apoptosis and apoptosis after 1 h, 3 h, 6 h administration. U937 cells ($1x10^6$ cells\ml) were treated at 12.5 μM for 1 h, 3 h and 6 h and then analysed through flow cytometry to detect apoptosis and necrosis levels. A: viable cells levels, B: early apoptosis levels, C: late apoptosis, D: necrosis levels. Data are presented as mean ± SD, n=3. **** ($P<0.0001$) using one way ANOVA Dunnett's post hoc test. Data are all compared to live control.

As shown in the previous section, it seemed logical to further investigate statistical data of the flow cytometry quadrants compared to the time course and not to live control. Therefore, the comparison between one hour of treatment and both three and six hours of treatment was considered. Also, the statistical differences between three and six hours were analysed. Interestingly, no relevant statistical difference between one hour and three hours in any of the cell conditions, apart from a $P<0.05$ difference for viable cells and early apoptosis cells at 50 µM. Also, when statistical data of one hour treatment were compared to six hours data, the only statistical differences were found again as $P<0.05$ on all conditions when cells were treated with 50 µM. Lastly, $P<0.05$ was found as a statistical difference on late apoptosis at 50 µM. All the others cell conditions were not significantly different. (Table 3.3.3.1.)

CELL CONDITION	1 HOUR VS 3 HOURS SIGNIFICANCE	1 HOUR VS 6 HOURS SIGNIFICANCE	3 HOURS VS 6 HOURS SIGNIFICANCE
12.5 µM viable cells	Not significant	Not significant	$P<0.1$
12.5 µM late apoptosis	Not significant	Not significant	Not significant
12.5 µM early apoptosis	Not significant	Not significant	Not significant
25 µM viable cells	Not significant	Not significant	Not significant
25 µM late apoptosis	Not significant	Not significant	Not significant
25 µM early apoptosis	Not significant	Not significant	Not significant
50 µM viable cells	$P<0.05$	$P<0.05$	Not significant
50 µM late apoptosis	Not significant	$P<0.05$	$P<0.05$
50 µM early apoptosis	$P<0.05$	$P<0.05$	Not significant

Table 3.3.3.1. **Further statistic comparing significance between the different times of administration of Pifithrin-µ on U937 cells**. The table shows the statistical differences between 1 hour and 3 hours administration, 1 hour and 6 hours administration and between 3 hours and 6 hours administration. All the flow cytometry quadrants were considered, apart from necrosis which have not been detected in previous analysis.

3.3.4. Heat shock protein PES-CL as single agent and its effect on cell viability of K562 and U937 cell lines following 24 hours administration

K562 cells, at a concentration of $1x10^6$ cells\ml, were administered with PES-CL and then incubated for 24 hours. Cell viability was analysed through MTS assay, as briefly described above. The choice of the range of PES-CL concentration was similar to what chosen with Pifithrin-μ; PES-CL was administered to K562 cells on a range from 50 μM to 1.5 μM. At 50 μM, only 8.29 % ($P<0.0001$) of the cells still maintained normal cell viability, showing a potential incredible effect. At 25 μM, there was still a significant effect, although different from 50 μM; 30.79 % of the cells ($P<0.0001$) were still alive. A decrease in the effect on cell viability was verified at 12.5 μM, where 55.27 % ($P<0.0001$) were not affected by PES-CL treatment. The remaining three concentrations did not affect cell viability; indeed 93.14 % of K562 cells were still alive at 6.2 μM and 90.03 % of cells were not affected by the drug at 3.1 μM. Lastly, at 1.5 μM, 92.07 % of cells did not result to have compromised viability (Fig. 3.3.4.1.)

U937 cells at a concentration of $1x10^6$ cells\ml, were administered with PES-CL and then incubated for 24 hours. Cell viability was assessed through MTS assay, as described in the methods section. Again, PES-CL was administered from a concentration of 50 μM to 1.5 μM. At 50 μM and 25 μM, the drug was extraordinarily effective with respect of cell viability; all U937 cells were killed by PES-CL when administered at these concentrations. At 12.5 μM, the drug was incredibly effective as well; only 2.95 % of the cells survived to the treatment. At 6.2 μM, there was a rapid increase to 49.75 % in the number of cells who have normal cell viability. At 3.1 μM, the majority of cells were not affected by PES-CL treatment; 88.24 % of the cells resulted to have a normal metabolism. Finally, 80.57 % of the cells survived the treatment at the lowest concentration, 1.5 μM (Fig. 3.3.4.2.)

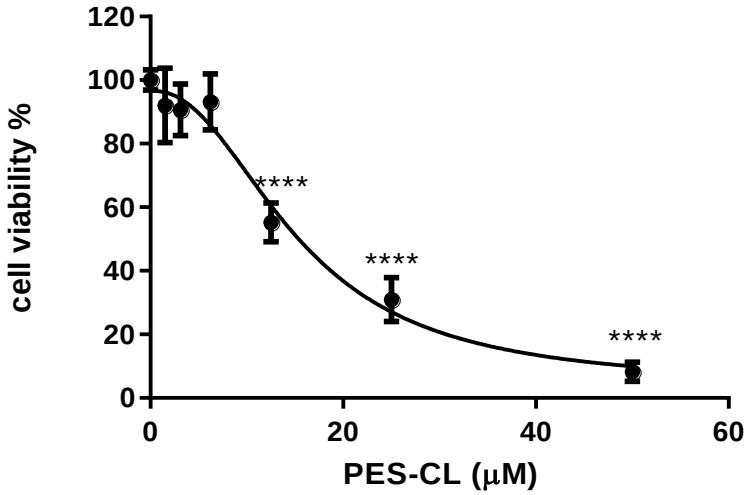


Fig 3.3.4.1. Effects on K562 cells (1x10^6 cells\ml) cell viability after 24 h treatment with PES-CL. After 24 h incubation, MTS assay was performed in order to measure cell viability. Data are presented as mean ± SD, n=4. **** (P<0.0001) using one-way ANOVA Dunnett's post hoc test. Data are all compared to live control and were normalized with dead cells control (not plotted). **IC_{50}: 15.62 μM.**

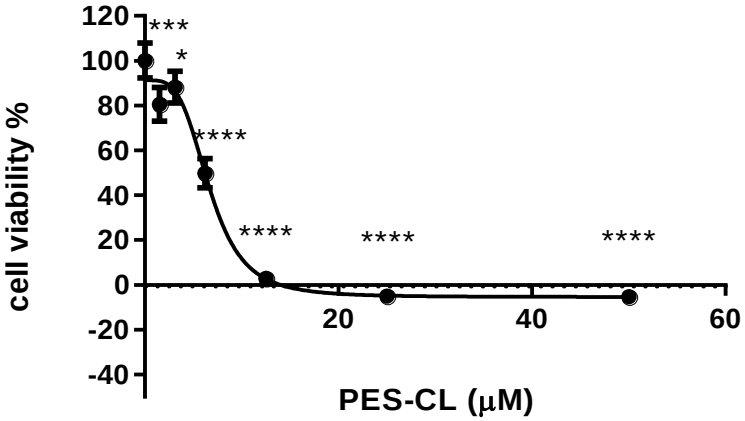


Fig 3.3.4.2. Effects on U937 cells (1x10^6) cell viability after 24 h treatment with PES-CL. After 24 h incubation, MTS assay was performed. Data are presented as mean ± SD, n=4. * (P<0.05), *** (P<0.001), **** (P<0.0001) using one-way ANOVA Dunnett's post hoc test. Data are all compared to live control and were normalized with dead cells control (not plotted). **IC_{50}: 6.7 μM.**

3.3.5. Heat shock protein PES-CL administered as single agent for up to six hours on U937 cells

After determining the effects of 24 h administration of PES-CL on U937 cells, a better understanding on the effects of such treatment for a shorter time of administration was needed. In the same fashion as what experimented with Pifithrin-μ, PES-CL was administered every hour up to six hours. Cell viability was measured by MTS assay as shown in the methods section. At 12.5 μM and following six hours of treatment, 44.11 % of the cells ($P<0.05$) did not result to have an affected cell viability. Five hours treatment resulted in 41.61 % of live cells ($P<0.05$), whilst four hours of treatment resulted in 47.93 % ($P<0.01$) of not affected cells. A similar result was accomplished following three hours of administration, where 48.54 % ($P<0.05$) of the cells survived the treatment. 58.02 % of the cells ($P<0.01$) were not affected by treatment following two hours of PES-CL administration, whilst 73.38 % of U937 were not as well affected following only one hour treatment (Fig. 3.3.5.1. A). At 25 μM and following six hours of treatment, 44.66 % of the cells ($P<0.05$) were still alive; following five hours of treatment, 45.64 % ($P<0.05$) of U937 cells were resistant to the treatment. After four hours of administration, 59.07 % of U937 cells did not respond to the treatment, followed by an increase of 66.33 % ($P<0.05$) in cell metabolic activity following three hours. The last two hours of treatment resulted in 53.08 % ($P<0.01$) of surviving cells after two hours of treatment and 54.24 % of them after one hour administration (Fig. 3.3.5.1. B). At the last concentration, 50 μM, six hours of treatment caused an important effect; only 21.38 % of the cells ($P<0.05$) were still live. Following five hours of treatment, 39.03 % of the cells ($P<0.01$) resulted to have a normal cell viability. From four hours to two hours, the cell metabolic activity detected by MTS was similar; 53.63 % ($P<0.05$) following four hours, 50.94 % following three hours and 52.97 % ($P<0.1$). There was an increase in normal cell viability detected following one hour only of treatment with PES-CL; metabolic activity was detected in 61.03 % of the cells (Fig. 3.3.5.1. C).

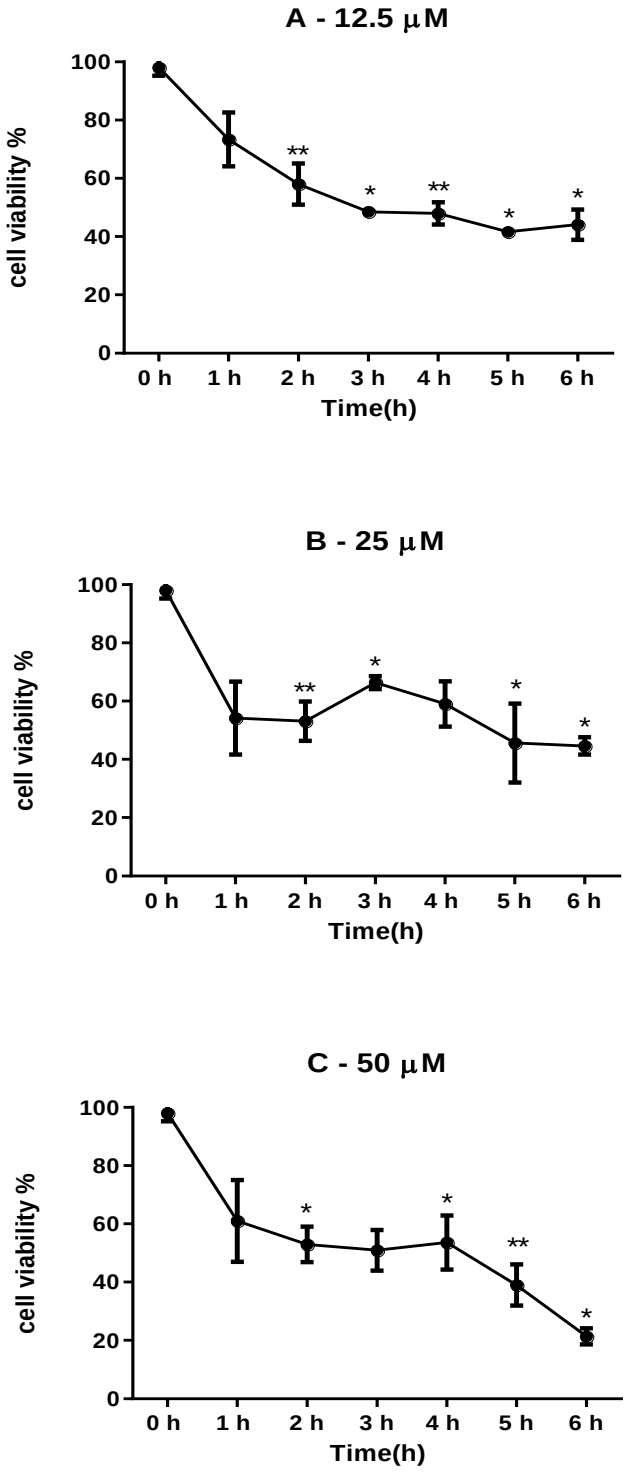


Fig 3.3.5.1. PES-CL administration up to 6 h followed by MTS assay on U937 (1x10^6). MTS solution was administered to cells after 6 hours of treatment; there is no significant difference between 12.5 μM (A), 25 μM (B) and 50 μM (C). The drug starts to be effective after 1-2 hours at all three concentrations. Data are presented as mean ± SD, n=3 * (P<0.05), ** (P<0.01), using one-way ANOVA Dunnett's post hoc test. Data are all compared to 0 h control and were normalized with dead cells control (not plotted).

3.3.6. Bortezomib as single agent and its effect on metabolic activity of K562 and U937 cell lines following 24 hours administration

Consistently with the other two drugs, the approach and the methods were the same with Bortezomib. MTS assay was used to determine metabolic activity levels on K562 cells and U937 cells on a 24 hours time course. Bortezomib, according to the manufacturer (Merck-Millipore), has been proved to be effective at much lower concentration than the two previous drugs; therefore, the range of concentrations started from 250 nM until 7.8 nM. Following 24 hours, 250 nM and 125 nM are effective on cell viability of K562 cells. Indeed, 35.06 % ($P<0.0001$) of the cells have a normal viability at 250 n M, whilst 125 n M resulted to not affect 44.63 % of the cells ($P<0.0001$). At 62.5 n M, the cell viability was not compromised for 47.23 % of the cell population. At 31.6 n M, 42.36 % ($P<0.0001$) was not affected by the treatment. A close result was accomplished at 15.6 n M, where 45.27 % of the cells were still alive ($P<0.0001$). An increase in cell viability has been found at 7.8 n M, where 74.08 % of cells was still alive (Fig 3.3.6.1). The IC_{50} calculated was 8.24 nM.

On U937, at 250 n M only 18.96 % ($P<0.0001$) of the cells were still viable following Bortezomib treatment. At 125 nM, the cells which maintained normal viability were 24.15 % ($P<0.0001$) whilst 21.94 % ($P<0.0001$) were still alive at 62.5 nM. A modest increase in cell viability was measured at 31.6 nM; indeed 24.36 % ($P<0.0001$) of the cells did not respond to treatment. Also, 33.36 % of the cells ($P<0.0001$) resulted viable at 15.6 nM, whilst cell viability increased to 49.14 % ($P<0.0001$) at 7.8 nM. Overall, none of the Bortezomib concentration administered to U937 killed less than 50 % of the cells. (Fig. 3.3.6.2.) The IC_{50} calculated was 5.45 nM.

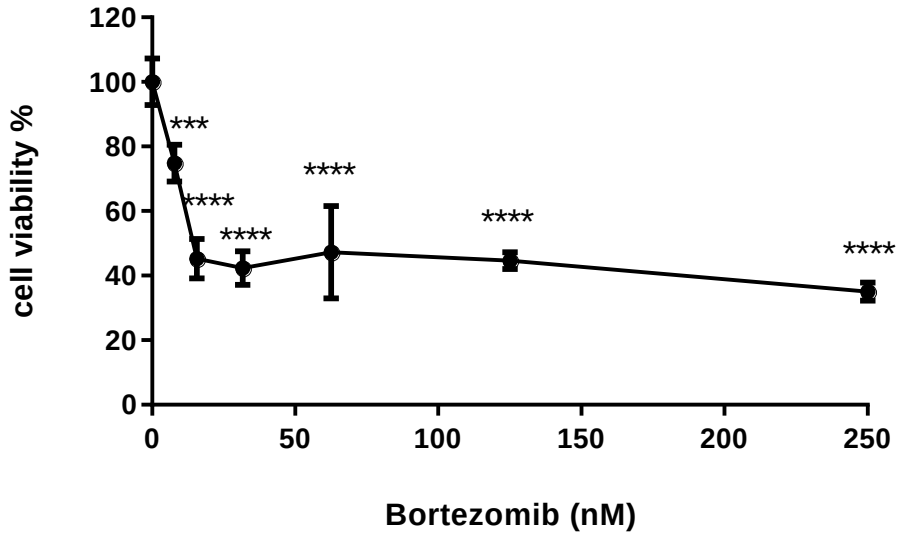


Fig 3.3.6.1. MTS assay of Bortezomib (250 n M) administered on K562 (1x10^{6}cells\ml). After 24 h incubation, MTS assay shows the cell viability. Data are presented as mean ± SD, n=3 *** (P<0.001), **** (P<0.0001) using one-way ANOVA Dunnett's post hoc test. Data are all compared to live control and were normalized with dead cells control (not plotted). **IC_{50}: 8.24 nM.**

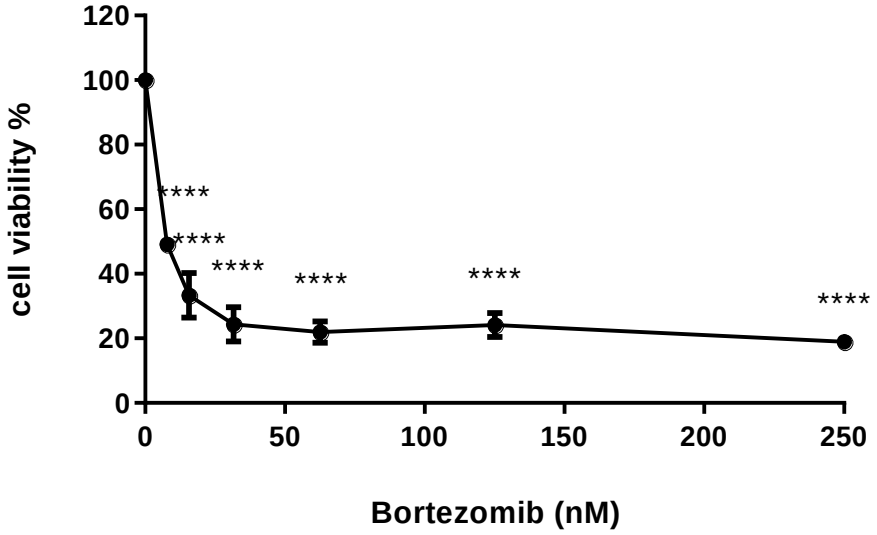


Fig. 3.3.6.2. MTS assay of Bortezomib (250 n M) administered on U937 (1x10^{6}cells\ml) After 24 h incubation, MTS assay assessed the cell viability. Data are presented as mean ± SD, n=3 **** (P<0.0001) using one-way ANOVA Dunnett's post hoc test. Data are all compared to live control and were normalized with dead cells control (not plotted). **IC_{50}: 5.45 nM.**

3.3.7. Bortezomib administered as single agent for one hour on K562 cells and U937 cells and subsequent determination of the type of cell death on the flow cytometry

The following step was to understand how Bortezomib kills the leukemic cells and when it starts to be effective in a 24 hours span. According to the literature and the drug producer, Bortezomib starts to be effective in 1 or 2 hours. Therefore, consistently with the experiments on HSPIs, the cells were treated with Bortezomib for 1 hour and then they were analysed on the flow cytometer following the Annexin V\PI protocol. The chosen drug concentrations were 31.2 n M and 15.6 nM, considering what was found in the previous MTS experiment. These were indeed the lowest concentrations which caused death to 50 % of K562 cells. On both concentrations, important levels of apoptosis have been detected; at 15 nM, 51.9 % of K562 cells died by early apoptosis and 5 % by late apoptosis (Fig. 3.3.6.1. Graph B). Also, at 31 nM the apoptosis percentage that was detected as 43 % early apoptosis and 5.1 % as late apoptosis following one hour only of Bortezomib administration (Fig 3.3.7.1. C).

Statistical data of the flow cytometry quadrants were also plotted and analysed. The graph was plotted in the same manner as the graphs designed for the HSPIs. Fig 3.3.7.2. shows that there was not any sign of necrosis on both concentrations following one hour of administration (D); also, late apoptosis has been calculated different to live cells control as ($P<0.01$) at 15.6 nM and as ($P<0.0001$) at 31 nM (C). Viable cells were analysed as statistically different as ($P<0.0001$) on both concentrations in comparison to live cells control (A). Also importantly, there is no statistical difference between the two concentrations following one hour administration of Bortezomib.

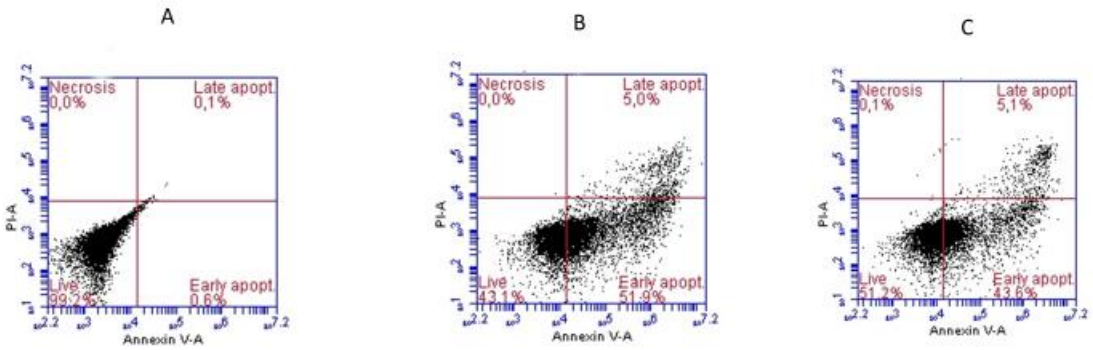


Fig. 3.3.7.1. Annexin V\PI assay of Bortezomib at 15.6 n M and 31.2 n M after 1 hour treatment on K562 cells. The drug was administered to cells at a $1x10^6$ cells\ml for 1 hour, followed by the analysis of necrosis and apoptosis levels. Respectively A: (untreated cells), B (15.6 nM), C (31.2 nM). Data are representative of one replicate, as an example. Overall the samples were analysed in triplicates for each treatment and for the control.

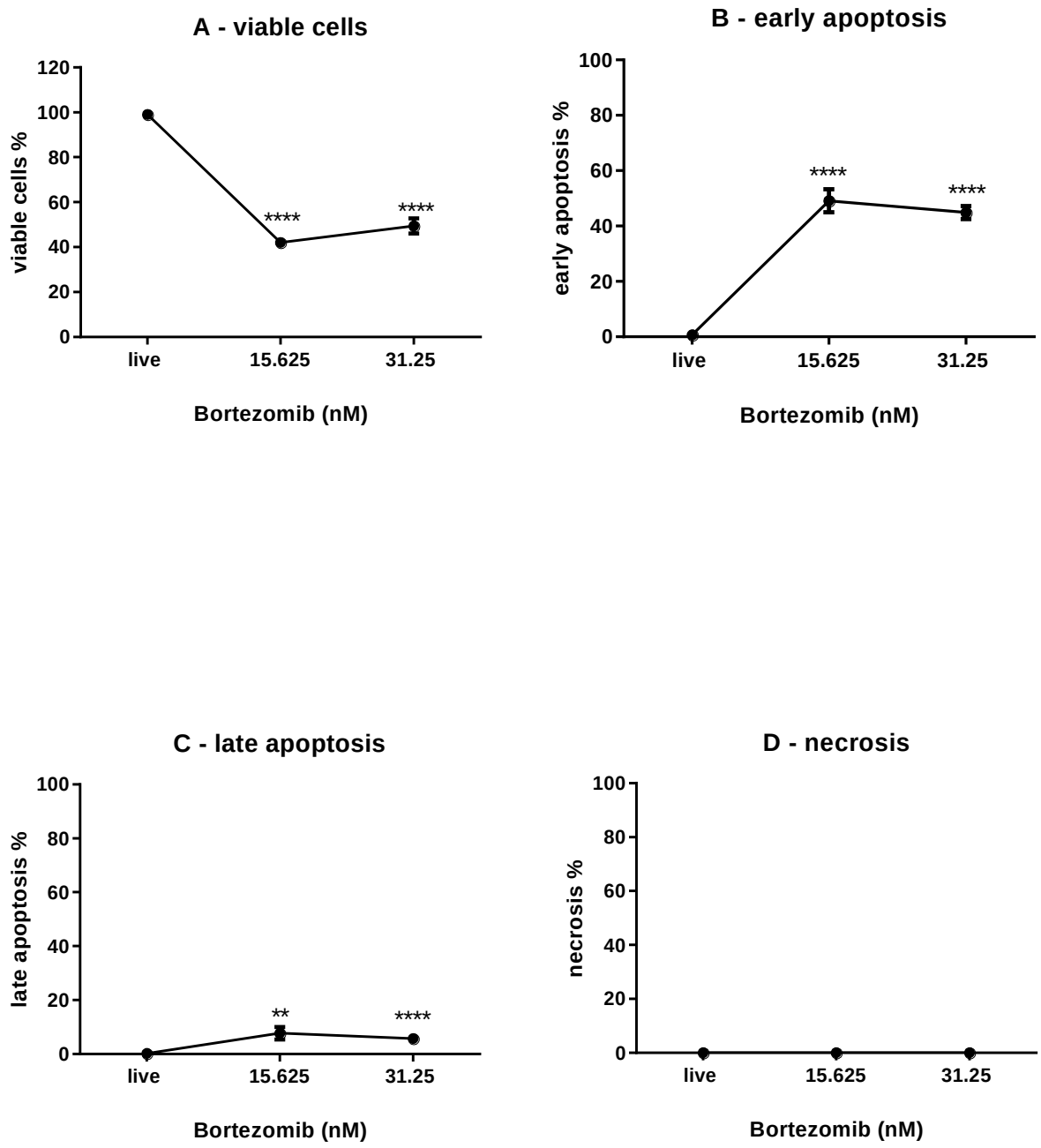


Fig 3.3.7.2. Effects of Bortezomib at 15.6 n M and 31.2 n M on K562 cells on viable cells, necrosis, late apoptosis and apoptosis after 1h administration. K562 cells ($1x10^6$ cells\ml) were treated at 15.6 n M and 31.2 n M for 1h and then analysed through flow cytometry to investigate the levels of necrosis and apoptosis. A: viable cells levels, B: early apoptosis levels, C: late apoptosis, D: necrosis levels. Data are presented as mean ± SD, n=6. ** (P<0.01), **** (P<0.0001) using one way ANOVA Dunnett's post hoc test. Data are all compared to live control.

The approach taken to determine what kind of death is caused by the administration of Bortezomib was the same of what described in the previous sections of this chapter. The concentrations chosen from the range tested on the MTS assay were 31.25 n M and 15.6 n M and the Annexin V\PI protocol was followed as described previously. Also, the drug was administered for only one hour, consistent with what experimented on K562 cells. Importantly, U937 was incredibly responsive to a short treatment; early apoptosis levels were detected as 66.4 % and late apoptosis as 11.4 % at 15 n M (Fig. 3.3.6.3. B), whereas 66.2 % of the cells were detected as early apoptosis and 15.5 % as late apoptosis affected by Bortezomib activity at 31.2 n M. (Fig. 3.3.7.3. C).

With respect of statistical data of the flow cytometry quadrants Fig 3.3.7.4 shows that there is no sign of cell death different from apoptosis (D); the difference between treated and live cells control in viable cells quadrant after one hour of treatment has been calculated as ($P<0.0001$) for both treatment (A). Percentage of late apoptosis were plotted also, indicating that there is no significative difference at 15.6 nM, but there is at 31.2 nM as $P<0.05$ (C) in comparison to live cells control. Early apoptosis levels showed that there is significative difference between treatments and live cells control analysed as ($P<0.0001$) at 15 nM and as ($P<0.0001$) at the following concentration, 31.2 nM (B).

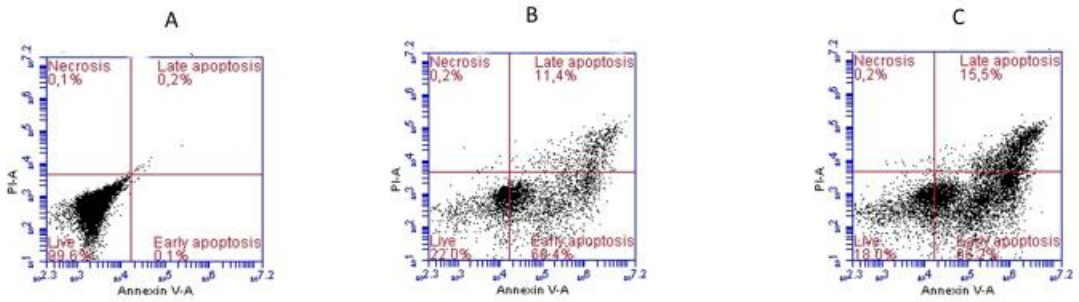


Fig. 3.3.7.3. Annexin V\PI assay of Bortezomib at 15.6 nM and 31.2 nM after 1 hour treatment on U937 cells. The drug was administered to cells at a $1x10^6$ cells\ml for 1 hour, followed by the analysis of necrosis and apoptosis levels. Respectively A: (untreated cells), B (15.6 n M), C (31.2 n M). Data are representative of one replicate, as an example. Overall the samples were analysed in triplicates for each treatment and for the control.

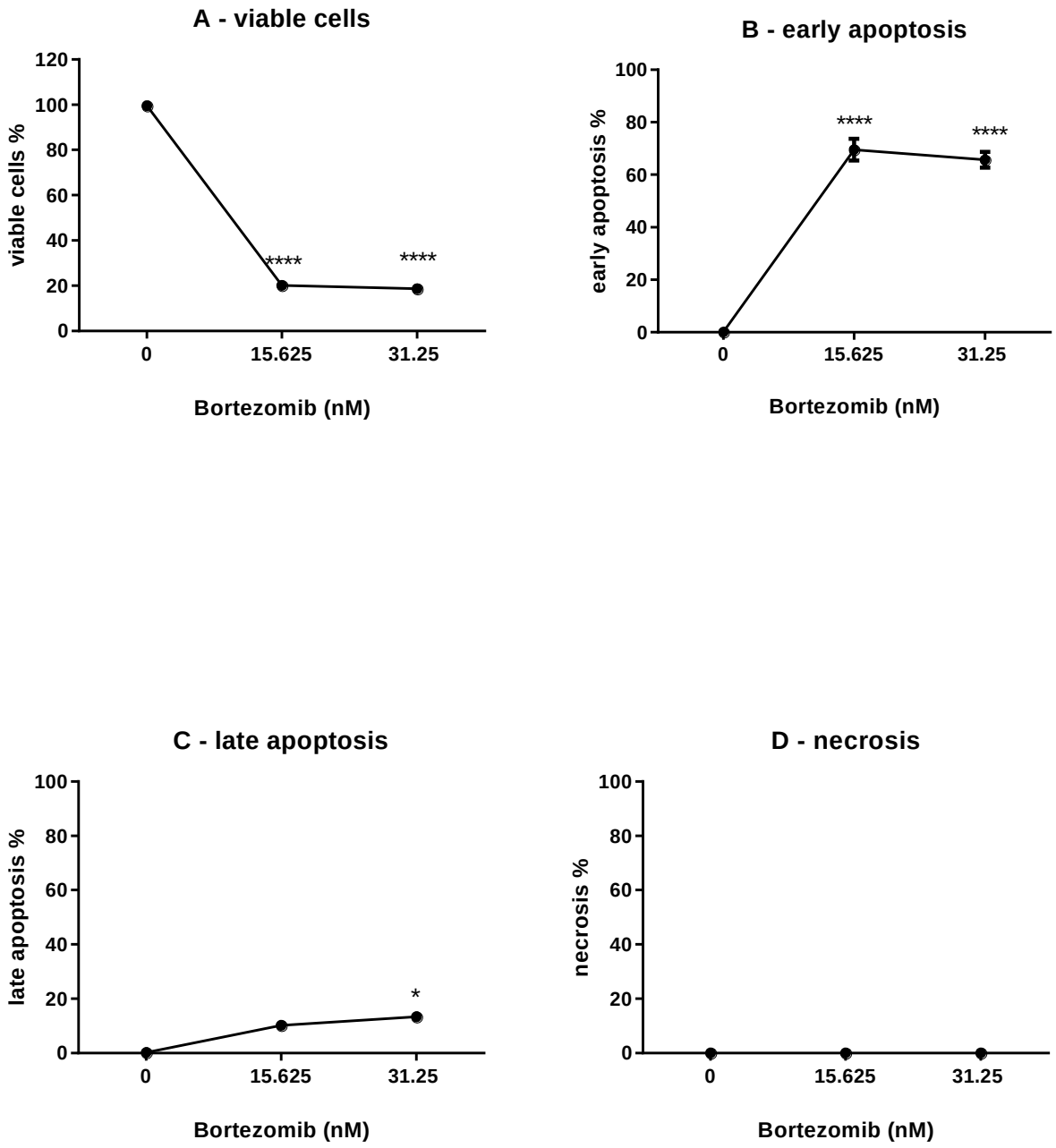


Fig 3.3.7.4. Effects of Bortezomib at 15.6 n M and 31.2 n M on U937 cells on viable cells, necrosis, late apoptosis and apoptosis after 1h administration. U937 cells ($1x10^6$ cells\ml) were treated at 15.6 n M and 31.2 n M for 1h and then analysed through flow cytometry to investigate the levels of necrosis and apoptosis. A: viable cells levels, B: early apoptosis levels, C: late apoptosis, D: necrosis levels. Data are presented as mean ± SD, n=6. *(P<0.05), **** (P<0.0001) using one way ANOVA Dunnett's post hoc test. Data are all compared to live control.

3.4. DISCUSSION

Leukemic cell lines K562 and U937 were administered with two heat shock protein inhibitors, which are Pifithrin-μ and PES-CL and a proteasome inhibitor, Bortezomib. They were all administered as single agents on both cell lines in order to assess cell viability and type of cell death in two different time course, one longer and one shorter.

3.4.1. Effects of Pifithrin-μ and PES-CL single agent treatments on cell viability on K562 and U937 cells

Pifithrin-μ has been administered to K562 cells and U937 cells from a range of concentration starting from 50 μM to 1.5 μM for a time of administration of 24 hours in order to verify its effects on cell metabolic activity through MTS assay. The drug showed a remarkable effect on cell viability at high concentrations, 50 μM, 25 μM and 12.5 μM. These concentrations affected more than 50 % of the cells after 24 hours of administration. The lower concentration, particularly 3.125 μM and 1.5 μM, were not particularly effective. These results differ from other studies; Kaiser *et al*, (2011) have demonstrated that Pifithrin-μ can have effect on cell viability at a lower concentration (8 μM). However, Pifithrin-μ was administered for 48 hours, whilst this chapter demonstrates that 12.5 μM has shown to be already effective after 24 hours on K562 cells. Moreover, it seemed also interesting to investigate the effects of this drug on a shorter time of administration, which was up to six hours of treatment. It seemed a sensible choice in a potential attempt to reduce the exposure to drugs to cells and, possibly, future patients. In fact, Pifithrin-μ started to be effective even in a shorter time than 24 hours. The range of concentration chosen for this experiment changed; the top three concentration that affected cell viability to more than 50 % of K562 and U937 cells were chosen. Six hours was a sufficient time to start affecting cell viability throughout the three different concentrations; the results were similar for both cell lines, considering that less than 50 % of the cells survived the treatment following six hours (Fig.3.3.2.1. and Fig.3.3.3.1.). Perhaps, the remaining live cells did not receive enough drug or the time course is too short; it is not yet clear how the drugs enter the cells, so this may need further evaluations. A study by Kanno, *et al*., (2015) partially supports the results of this chapter; Pifithrin-α, a progenitor of Pifithrin-μ, demonstrated that 4 hours of treatment with 20 μM Pifithrin-α are able to significantly suppress cytochrome c release in hepatocellular carcinoma HepG2 cells. Although it is not the same drug, this can suggest that Pifithrin-μ mechanism of action may occur in a short time, as shown in this chapter. Same pattern was found after five and four hours, again similarly between the two cell lines. These results are partially similar to what found by Monma H. *et al*. (2013), who demonstrated that Pifithrin-μ affects cell viability on four different cell lines following 5 hours of administration; however, these four cell lines are pancreatic cancer cell lines, whilst this study analyses the effects on leukemic cell lines.

Similar to Pifithrin-μ experiments, the range of PES-CL concentrations was reduced to the top three concentrations and MTS assay was again used to assess cell metabolic activities. The assay showed that six hours of administration can affect more than half of the cells at 50 μM, trend that continued when the time of administration decreased; at one hour of administration, there was still an effect on cell viability (Fig.3.3.5.1.). This partially agree with a study where PES-CL has been shown to arrest cell cycle in a short time (6 hours), on colorectal and small lung lymphoma cells at 20 μM (Balaburski G. *et al*, 2013). The effects on U937 cells metabolism were similar, indeed at different times of administration at least half of the cells had their metabolic activity affected, for example after six hours and two hours. Importantly, after one hour there was still a considerable effect on cell metabolic

activity, where slightly less than 50 % of the cells were affected by PES-CL. More notably, 12.5 µM showed to have effects on cell viability following a short time of administration. On contrary to what found by (Balabursky G. *et al*., 2013) a smaller concentration than 20 µM is sufficient to start affecting cell viability to more than half of the cells after six, five and four hours. The last three hours still resulted in affected cell viability, confirming that a short time of administration is sufficient to trigger apoptosis.

Both drugs showed that 12.5 is the lowest concentration to affect cell viability on both cell lines. However, the drugs killed almost half of the cells population; considering that these were single agent experiments, these results could be considered significant. Moreover, it is important to note that the lowest concentration would be added to another drug, Bortezomib, in an attempt to improve single agent results.

3.4.2. Effects of Pifithrin-µ single agent treatments on apoptosis on K562 and U937 cells

The results given by MTS assay led to the investigation of the type of cell death that occurred on both cell lines. Annexin V\PI was the assay on the flow cytometer that was chosen to detect the kind of cell death. Importantly, the drug was administered after six, three and one hour; the reason was that there was no difference statistically between six hours until three hours and from three hours to one hour on both cell lines. The cells died by early apoptosis throughout the whole set of concentrations on both cell lines (Fig.3.3.2.2. and Fig.3.3.3.2.). There was no sign of necrosis, supporting what found in various studies (Guo F. *et al*, 2005 & Sekihara K. *et al*, 2013). Perhaps the reason why the cells died by apoptosis and not by necrosis could be explained by the fact that HSP70 is involved in the inhibition of the mitochondrial pathway of apoptosis by blocking Apaf 1 by repressing the activity of caspase 3 and 9 on leukemic cells (Gurbuxani S. *et al*, 2003). Possibly, Pifithrin-µ may restore the mitochondrial pathway of apoptosis. Statistically, the different cell conditions were plotted to investigate significant differences between the different times of drug administration; generally, there was no significative difference in respect of early apoptosis between one hour and three hours and one hour and six hours, excluding at 50 µM for U937. On K562, there was no significant difference between one hour and three and one hour and six hours on any drug concentration, excluding at 50 µM when three and six hours were compared (Table 3.3.2.1.) It is important to note the particular relevance of these findings, especially for 12.5 µM on both cell lines. Indeed, the lowest concentration could start triggering apoptosis following one hour and there is no difference in terms of drug administration between one hour and three hours and one and six hours. Although in combination with another drug, the results of this chapter find partial confirm on a study by Krukowski, *et al*., (2015). Pifithrin-µ has been demonstrated to enhance cell death together with Paclitaxel when Pifithrin-µ is administered for one hour before administering Paclitaxel, preventing chemotherapy-induced peripheral neuropathy (Krukowski, K., *et al*., 2015).

3.4.3. Effects of Bortezomib single agent treatments on cell viability and apoptosis on K562 and U937 cells

The range of concentration started from 250 nM to 7.8 nM and the drug was administered for 24 hours, equally to what performed to the two HSPIs. MTS assay showed that Bortezomib affected cell viability to both cell lines to more than 50 % of K562 cells and to more than 60 % of U937 cells, throughout the entire range of concentration, excluding 7.8 nM, after 24 hours treatment (Fig.3.3.6.1. and Fig.3.3.6.2.). These results partially disagree with other studies (Klikova K. *et al*, 2015) where K562 cells viability has been affected after 72 hours and not 24, as this thesis demonstrates. Interestingly, Bortezomib has been administered in combination with several other chemotherapy drugs, such as cytarabine (Attar E.C. *et al*, 2013) on this cell line; however, the results of this chapter indicate a potential use of the drug as single agent at low concentrations. Further, it was necessary to determine the type of cell death and if the mechanism of action of Bortezomib effectively starts to trigger the cell death on a shorter time than 24 hours. To better understand and evaluate the type of cell death, the concentrations analysed were 15.6 n M and 31.2 n M, which were the lowest concentration to affect cell viability, according to MTS assay. The cells were administered with Bortezomib for 1 hour and then analysed on the flow cytometer. With respect of U937, the flow cytometry results also confirmed the effectiveness found by the MTS assay; more than 60 % died by early apoptosis, following one hour of administration on both concentrations. Similar results have been found for the treatment of retinoblastoma cells (Poulaki V. *et al*, 2007), but apoptosis was not triggered within 1 hour, differing from what found in this chapter. No significant difference between 15.6 nM and 31.2 nM was calculated, indicating that 15 nM could be a potential candidate to kill acute leukemic cells following one hour administration.

These results can give an important first insight on how these three drugs behave in the treatment of CML and AML; although administered only as single agents, these three drugs affect significantly cell viability and trigger apoptosis in a very short time. Future experiments may aim to further understand how the drugs enter the cells and therefore potentially improve the effectiveness of the drugs and possibly reduce the doses. These results will be then considered for the following chapter, which investigates the effectiveness of these three small molecules combined.

CHAPTER 4: ARE THE HEAT SHOCK PROTEINS INHIBITORS AND BORTEZOMIB ANTAGONISTIC OR SYNERGISTIC ON LEUKAEMIA CELL LINES?

4.1. INTRODUCTION

This chapter explores the effects on cell viability and apoptosis of combination treatment between Heat Shock Protein Inhibitors (HSPIs) and the proteasome inhibitor Bortezomib on two leukemic cell lines, which are U937 and K562. Combined therapy is widely used as a treatment option in cancer in general. The use of a drug as single agent may be effective enough to kill cancer cells, but the combination therapy can target different key pathways resulting in higher chances to obtain apoptosis induction or reduction of drug resistance (Mokhtari, R. *et al*., 2017). Moreover, drugs combination could result in a synergy effect or antagonistic effect; synergy can occur when the effects of the drugs combined are overall greater than the drug effects alone. Therefore, antagonism occur when the effects of drugs combined are not able to overcome the results obtained when the drugs are administered alone. The choice of new potential combined therapy depends strongly on the eventual synergy of the drugs, therefore it is paramount to investigate the effects of the drugs as single agents and then combine them to exclude the antagonistic effect. However, the potential synergy cannot explain the possible mechanism for drug combinations (Chen D.*et al*., 2015).

Pifithrin-μ seems to enhance the activity of different chemotherapeutic drugs, such as cytarabine, 17-(allylamino)-17-desmethoxygeldanamycin, suberoylanilide hydroxamic acid, and sorafenib (Kaiser *et al*, 2011). These molecules all belong to the neoplastic drugs family and they have separate and different targets; cytarabine targets specifically the S phase of the cell cycle, inhibiting the DNA synthesis. 17-(allylamino)-17-desmethoxygeldanamycin is a HSP90 inhibitor, which directly binds to this protein and promotes apoptosis; the mechanism of action is still not completely understood (Ochel, H.-J., *et al*., 2001). Suberoylanilide hydroxamic acid or Vorinostat is responsible the deacetylation of lysine residues of histones, which induces apoptosis due to the accumulation of acetylated histones (Leoni F. *et al*, 2002). Lastly, Sorafenib is a small molecule inhibitor which simultaneously affects the Raf/Mek/Erk pathway (Llovet M.J. & Hernandez-Gea, V., 2014). As said above, the combination between Pifithrin-μ and these drugs, and consequentially, the aim to target different pathways, can enhance the levels of apoptosis on AML and ALL cell lines. Further, Pifithrin-μ could play a role in the improvement of the effects of Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) on pancreatic cancer cells. Considering that in cancer cells the resistance therapy may unfortunately happen, an important option to improve sensitivity to drugs is the inhibition of autophagy. TRAIL has been reported to successfully affect the mechanisms of autophagy; HSP70 also may be involved in the attempt to restore normal levels of autophagy. Also, TRAIL and Pifithrin-μ seems to target a common pathway, although at different stages: the NF- κB pathway. Therefore, it has been demonstrated that Pifithrin-μ can assist drugs like TRAIL to increase cancer cells cell death; more importantly, the combination therapy resulted in a decreased pancreatic tumor growth if compared with results obtained by a single agent treatment (Monma H., *et al*., 2013). Pifithrin-μ also appeared to play a role in preventing Chemotherapy-induced peripheral neuropathy (CIPN), which is a common side effect of cancer treatment. Although it is not completely clear, CIPN occur when there is a discontinued treatment of compounds like paclitaxel. In vivo, CIPN

occur in presence peripheral mechanical allodynia in association with retraction of intraepidermal nerve fibers (IENFs). Although the causes of CIPN are not fully clear, it has been demonstrated that mitochondrial dysfunction could affect the regulation of peripheral sensory neurons. *In vitro*, Pifithrin-μ was administered prior the treatment with paclitaxel on cancer ovarian cells, to try to investigate if the involvement of HSP70 and p53 may contrast the paclitaxel cytotoxic effect. The combined therapy not only did not prevent the effects of paclitaxel, but enhance the apoptosis rate in the ovarian cells (Krukowski K., *et al*., 2015).

There is not enough knowledge about PES-CL mechanism of action and its effects on a combined therapy; however, it seems that PES-CL and Pifithrin-μ may be able to enhance the apoptosis induction in melanoma patients when they were treated with BRAF inhibitors. Particularly, it appears that the treatment with HSP70 inhibitors such as Pifithrin-μ and PES-CL may overcome the resistance to BRAF inhibitors (Budina-Kolomets A. *et al*, 2016). The mechanism of action of PES-CL is still not completely understood, whether it has been tested as single agent or in combination with other drugs; further studies are needed and, perhaps, this study could help investigating how this drug may be effective in leukaemia treatment.

Bortezomib is a proteasome inhibitor which has been extremely successful in multiple myeloma treatment, as a single agent or in combined therapy. A study by (Saha M.N., *et al*., 2010) have demonstrated that Pifithrin-μ plays an important role when it is combined with Bortezomib and Nutlin, which is a small molecule that inhibits p53. Interestingly, Pifithrin-μ was administered to multiple myeloma cells and following six hours Nutlin and Bortezomib were added to the cells. Pifithrin-μ and Nutlin seems to induce apoptosis following this time of treatment; however, Pifithrin-μ due to its specificity to p53, induced apoptosis in a p53 dependent fashion. When Nutlin was administered with Bortezomib only or with Pifithrin-α, the apoptosis was induced independently from the mitochondrial pathway, where p53 is deeply involved. Also, it has been demonstrated that Pifithrin-μ and Bortezomib could synergically affect cell viability in B-cell lymphoma. Although the mechanism is not clear, it seems that 16 hours of combined treatment may be able to affect the viability of BC3 and BCBL1 cells. Granato M. *et al*. (2013) have suggested that Pifithrin-μ may possibly enhance proteasome inhibitor activity overcoming drug resistance. However, they also suggested a possible antagonistic effect between the two drugs when HSP40, HSP72, HSP90 and HSP25 activities were investigated; therefore, the involvement of HSP70 in a possible antagonistic effect between HSPIs and proteasome inhibitors demonstrates to be still not completely clear (Granato M. *et al*, 2013). Moreover, the role of these HSPs in proteasome modulation and protection from inhibition was suggested to be independent of ATP, indicating a potential novel mechanism for HSP70 action, opposite to what is commonly believed (Rodriguez, K. A., *et al*, 2014). Further studies may perhaps elucidate this mechanism and its role in the triggering of apoptosis. An interesting confirm that Bortezomib may be p53 dependant has been given by Wang C. *et al*, (2012). This study suggested that Bortezomib combined with rapamycin induces apoptosis and suppress cell growth on hepato-carcinoma cell lines; also, the effects on tumour suppression on mice were superior (72.4%) than when the animals were treated with the drug alone (rapamycin effects were 54.7 % and after bortezomib only they were 22.4%). Importantly the treatment with Pifithrin-α showed that cell apoptosis was importantly affected, suggesting that Bortezomib effect may be dependent from p53 accumulation in the cytoplasm. However, Bortezomib has also been demonstrated to be effective in the treatment of neuroblastoma. Indeed, due to its role in angiogenesis and in cell growth already documented on neuroblastoma, Bortezomib was combined with All-trans retinoic acid (ATRA).

Bortezomib showed to enhance the effects of ATRA on cell proliferation by increase the neuronal differentiation effect of ATRA on neuroblastoma cell lines, showing also a reduced toxicity when the combined therapy was repeated in vivo (Luo, P., *et al*., 2011).

Considering the mechanisms of action of these two families and the results of single agents described on Chapter 3, combined experiments were performed and therefore shown on this chapter. This was reflected in the time course choice; 24 hours of combined treatment seemed a sensible choice considering that the drugs were effective following this time course on both cell lines. Also, these results were partially supported by other studies, as described in the previous paraghraps. More importantly, considering that the three drugs showed to affect cell viability and induce apoptosis following just one hour of treatment, it was decided to administer a drug for one hour and then add subsequently the other drug for 24 hours. In fact, this protocol intended to obtain an initial effect with the first drug and then a potential enhancement with the second drug over a 24 hours time course. The scheme below sums the protocol just described.

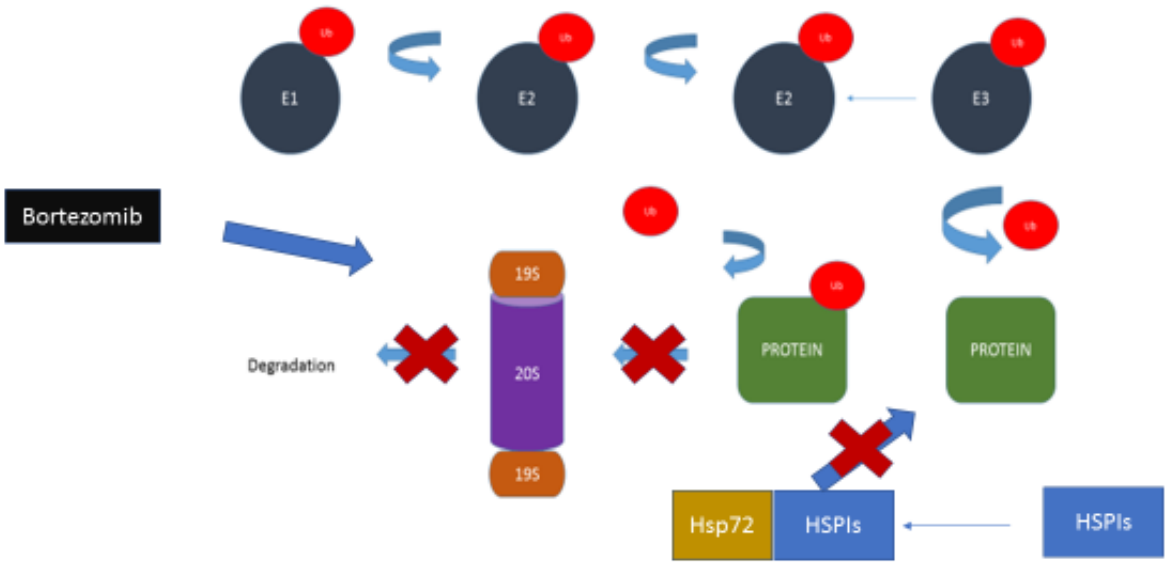


Fig. 4.1.1. Combination experiments rationale scheme. The two drugs, Bortezomib and one of the HSPIs, were administered on leukemic cell lines in combination. When Bortezomib was administered first for one hour, it is believed it bound to the proteasome, avoiding the degradation of pro-apoptosis proteins. The subsequent addition of one of the HSPIs after one hour aimed to target the remaining pro-survival proteins by inhibiting Hsp72 in his chaperone action to the proteasome. The same rationale was considered for when the HSPIs were added first for one hour, followed by 24 hours treatment with Bortezomib.

The aims of this chapter are:

- Investigate the synergistic or antagonistic effects on cell viability of 24 hours of combined therapy between Pifithrin-μ and Bortezomib on K562 cells and U937 cells.
- Investigate the synergistic or antagonistic effects on cell viability of 24 hours of combined therapy between PES-CL and Bortezomib on K562 cells and U937 cells.
- Understand the type of cell death following the different combined therapies.

4.2. METHODS

4.2.1 Cell culture

K562 and U937 cell lines were used for these set of experiments. The cells were cultured and subdivided as described in Chapter 2.3.1. Both cell lines were treated at a $1x10^6$ cells\ml concentration.

4.2.2. Choice of concentrations and treatment

The drugs used for the experiments in this chapter are two HSPIs (Pifithrin-μ and PES-CL) and the proteasome inhibitor Bortezomib. The drugs were diluted with DMSO according to what described on Chapter 2.2.6. Considering the results of single agent experiments showed in chapter 3, it was decided to choose the minimum dose which affected cell viability for a period of treatment of 1 hour up to 6 hours. HSPIs dose was 12.5 μM and Bortezomib doses were 15.6 n M and 31.2 n M. The cells were then treated as described on Chapter 2.3.5.2. The different combination experiments are listed in the table below, including the technique used. (Table 4.2.1.).

COMBINATION	TECHNIQUE	CELL LINE
1 h PES-CL\24 h Bortezomib	MTS	K562
1 h Pifithrin-μ\24 h Bortezomib	MTS	K562
1 h Pifithrin-μ\24 h Bortezomib	MTS	U937
1 h Bortezomib\24 h Pifithrin-μ	MTS	U937
1 h PES-CL\24 h Bortezomib	MTS	U937
1 h Bortezomib\24 h PES-CL	MTS	U937
1 h Pifithrin-μ\24 h Bortezomib	Flow cytometry	K562
1 h PES-CL\24 h Bortezomib	Flow cytometry	K562
1 h Bortezomib\24 h PES-CL	Flow cytometry	K562
1 h PES-CL\24 h Bortezomib	Flow cytometry	U937
1 h Bortezomib\24 h PES-CL	Flow cytometry	U937
1 h Pifithrin-μ\24 h Bortezomib	Flow cytometry	U937
1 h Bortezomib\24 h Pifithrin-μ	Flow cytometry	U937

Table 4.2.2.1. List of combination experiments with HSPIs on K562 and U937 cell lines. The combination experiments present on this table are listed according to the technique and to the cell line. Importantly, the experiments are listed in the exact order as presented in the result section.

4.2.3. MTS assay

Following the 24 hours treatment, MTS assay was performed as described on Chapter 2.3.6.

4.2.4. Annexin V\PI assay

Following the 24 hours treatment, the cells were prepared and analysed as described on Chapter 2.3.7.1. – Annexin V\PI assay.

4.2.5. Combination index analysis

The analysis was performed according to what described on Chapter 2.3.8.

4.2.6. Statistical analysis

All the statistical analysis were performed according to what described on Chapter 2.3.9.

4.3. RESULTS

4.3.1. Effects on cell viability on K562 cells following 1 hour treatment with HSPIs and subsequent 24 hours treatment with Bortezomib.

After 24 hours MTS assay showed that PES-CL is effective by affecting cell viability when added as single agent to K562 cells; only 20.48 % of the cells were still viable at 12.5 µM. When Bortezomib was added following one hour, the cell viability levels were increased. When combined with PES-CL, Bortezomib was equally effective at 15.6 nM and 31.2 nM; 36.16 % and 37.22 % of cells, respectively, were not affected by the treatment. When Bortezomib was added to K562 cells following one hour, but as single agent, resulted in 57.71 % of cells still viable at 15.6 nM and in 47.29 % of cells at 31.2 nM. All the drugs combinations were found significantly different as $P<0.0001$. (Fig. 4.3.1.1.)

When Pifithrin-µ is administered for 24 hours on K562 at 12.5 µM, cells were responsive to treatment. Pifithrin-µ as single agent resulted in 16.74 % of K562 cells with normal cell viability. Following one hour of treatment with Pifithrin-µ cells were treated with Bortezomib as single agent or added to Pifithrin-µ; when they were treated with Bortezomib after one hour, 28.37 % of K562 cells did not respond to treatment when Bortezomib was at 15.6 nM and 25.39 % of K562 cells when 31.2 nM Bortezomib was added to Pifithrin-µ treated cells. All the drugs combinations were found significantly different as $P<0.0001$. (Fig. 4.3.1.2.)

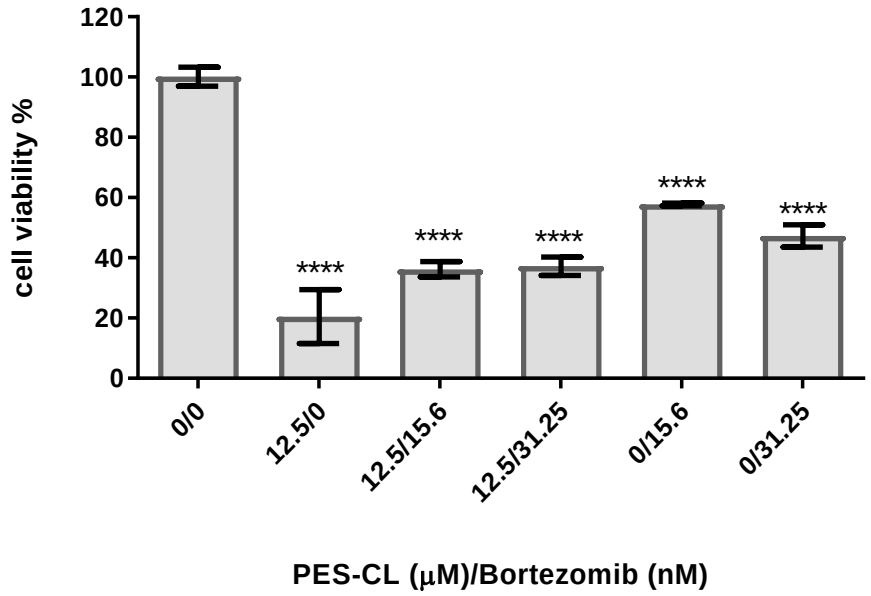


Figure 4.3.1.1. Effects on K562 cells (1x10^6 cells\ml) cell viability after 1 h PES-CL treatment and subsequent 24 h treatment with Bortezomib. After 24 h incubation, MTS assay was performed. Data are presented as mean ± SD, n=3. **** (P<0.0001) using one-way ANOVA Dunnett's post hoc test. Data are all compared to live control.

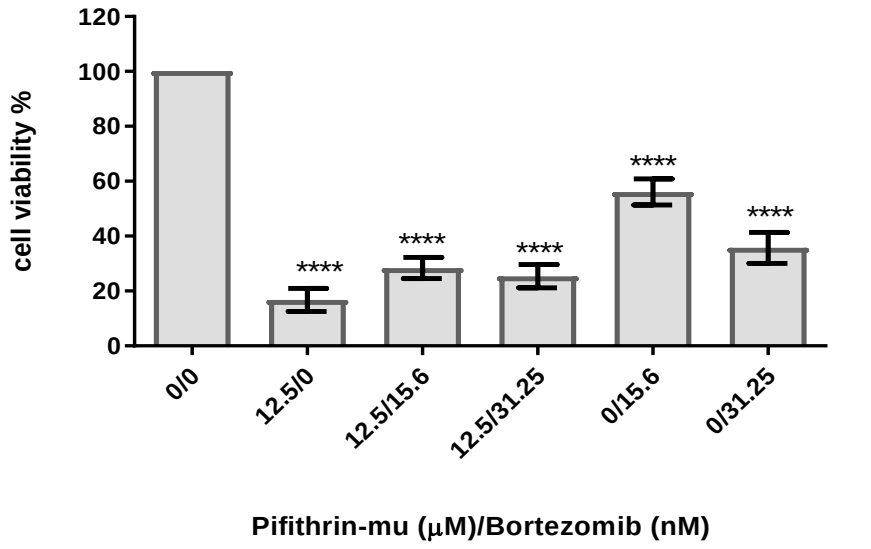


Figure 4.3.1.2. Effects on K562 cells (1x10^6 cells\ml) cell viability after 1 h Pifithrin-μ treatment and subsequent 24 h treatment with Bortezomib. After 24 h incubation, MTS assay was performed. Data are presented as mean ± SD, n=3. **** (P<0.0001) using one-way ANOVA Dunnett's post hoc test. Data are all compared to live control.

4.3.2. Effects on cell viability of Pifithrin-μ and Bortezomib combined treatment for 24 hours on U937 cells

With respect of U937, Pifithrin-μ resulted in 23.42 % of not affected cells when administered as single agent at 12.5 μM for 24 hours. Similar to K562 cells, when 15.6 nM Bortezomib was added to the cells previously treated with Pifithrin-μ, there was an increase in cell viability levels, 31.18 %. The cell viability levels decreased again to 26.91 % when 31.2 nM of Bortezomib was added after 1 h treatment of Pifithrin-μ. When Bortezomib was administered as a single agent, 51.24 % of U937 cells were not affected by 15.6 n M alone. When Bortezomib was added as 31.2 n M, only 27.34 % of the cells were still viable. Statistically, the combinations Pifithrin/Bortezomib were all significant different from live control. When all the possible drug combinations were compared together, 12.5/15.6 compared to 12.5/31.25 was significant different; same statistic difference was found when 12.5/15.6 was compared to 31.2 n M. All the drugs combinations were found significantly different as $P<0.0001$. (Fig. 4.3.1.1.)

When Bortezomib was added for 24 hours, it had an effect of U937 cells. As single agent, only 28.57 % ($P<0.0001$) of U937 cells survived 24 hours treatment of 15.6 n M. Even less cells were not affected by 31.2 n M after 24 hours, 21.28 % ($P<0.0001$). When Pifithrin-μ was added after one hour of Bortezomib treatment, it had a strong effect on cell viability, indeed 28.17 % ($P<0.0001$) of U937 cells were not affected by Pifithrin-μ. When Pifithrin was added to U937 cells previously treated for one hour, 50 % of cells were not affected at 12.5\15.6, whilst the cell viability was more compromised when Pifithrin was added to 31.2 n M, where 32.43 % of cells were not compromised ($P<0.0001$). Statistically, only 12.5/15.6 when compared to 31.25 alone is significantly different ($P<0.0001$). These results are shown on Fig. 4.3.2.2.

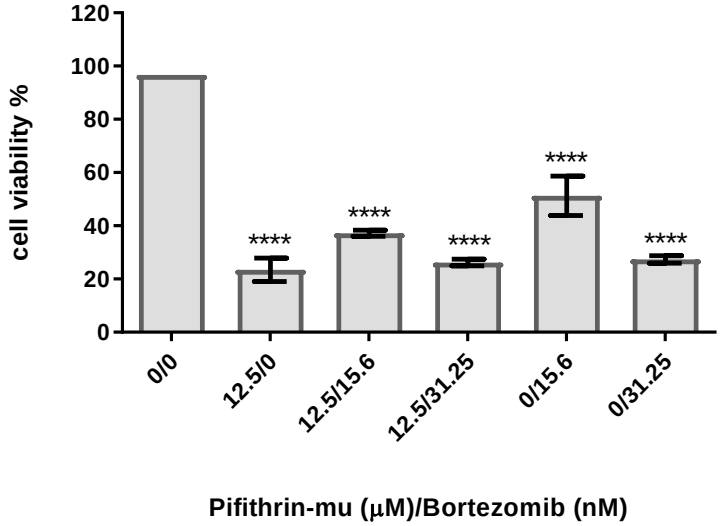


Figure 4.3.2.1. Effects on U937 cells (1x10^6 cells\ml) cell viability after 1 h Pifithrin-μ treatment and subsequent 24 h treatment with Bortezomib. After 24 h incubation, MTS assay was performed. Data are presented as mean ± SD, n=3. **** (P<0.0001) using one-way ANOVA Dunnett's post hoc test. Data are all compared to live control.

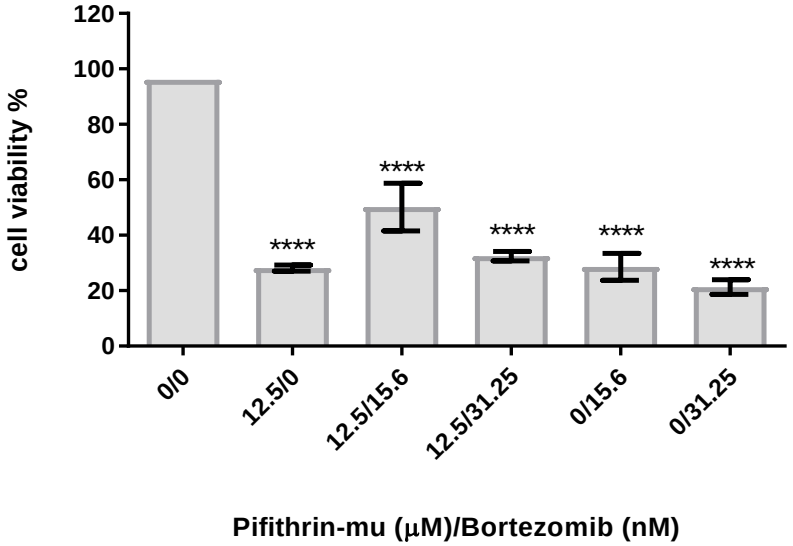


Figure 4.3.2.2. Effects on U937 cells (1x10^6 cells\ml) cell viability after 1 h Bortezomib treatment and subsequent 24 h treatment with Pifithrin-μ. After 24 h incubation, MTS assay was performed. Data are presented as mean ± SD, n=3. **** (P<0.0001) using one-way ANOVA Dunnett's post hoc test. Data are all compared to live control.

4.3.3. Effects on cell viability of PES-CL and Bortezomib combined treatment for 24 hours on U937 cells

On U937 cells treated with 12.5 µM PES-CL for 24 hours, the cell viability was importantly affected. When PES-CL was added to the cells as a single agent, only 2.43 % of cells survived the 24 h treatment ($P<0.01$). Following 1 h treatment with PES-CL, U937 cells were treated with Bortezomib at 15.6 n M and at 31.2 n M. At 12.5\15.6, 12.68 % ($P<0.0001$) of cells were not affected by the treatment; an equal result was found when 31.2 n M was added to cells which were treated for one hour with PES-CL. Indeed, 12.68 % ($P<0.0001$) of cells were not affected by the combination of drugs. Consistently with the results shown before, Bortezomib as single agent is less effective, especially at 15.6 n M. At this concentration, 40.56 % ($P<0.0001$) were still viable after 24 hours of Bortezomib treatment. At 31.2 n M, 24 hours treatment resulted in 22.30 % ($P<0.0001$) of U937 cells with not compromised cell viability. Statistically, all the combinations resulted different from the controls. When the concentrations were all compared, it was found that 12.5 µM is different from 15.6 n M and 31.2 n M as ($P<0.0001$); also, 15.6 n M was different as ($P<0.0001$) when compared to combined treatment, therefore 12.5\15.6 and 12.5\31.2 (Fig. 4.3.3.1.)

When Bortezomib is added to U937 cells for an hour, prior PES-CL treatment, there is also an effect on cell viability. At 15.6 n M single agent, 23.70 % ($P<0.0001$) of cells were still viable, whilst 19.43 % ($P<0.0001$) of the cells did not have an affected metabolism when Bortezomib is at 31.2 n M. When PES-CL is added as a single agent one hour following Bortezomib treatment, U937 cells responded incredibly to the treatment. Only 4.02 % ($P<0.0001$) of cells were not affected by the treatment; instead, when PES-CL was added to U937 cells which received 1 hour of Bortezomib treatment, the cell viability levels increased. At 12.5\15.6, 32.92 % ($P<0.0001$) of cells had a not affected metabolism, while when PES-CL was added to 31.2 n M treated cells the percentage of surviving cells was 19.12 % ($P<0.0001$). Statistically, the drug concentrations were all different from controls. When they were compared between each other, 12.5 µM resulted different as ($P<0.0001$) when compared to 15.6 n M Bortezomib single agent and as ($P<0.0001$) when compared to 12.5\15.6. (Fig. 4.3.3.2.).

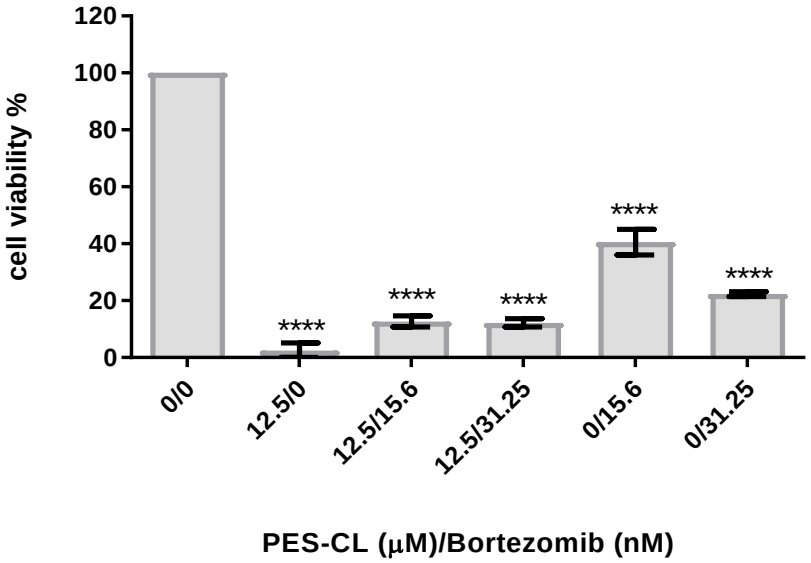


Figure 4.3.3.1. Effects on U937 cells (1x10^6 cells\ml) cell viability after 1 h PES-CL treatment and subsequent 24 h treatment with Bortezomib. After 24 h incubation, MTS assay was performed. Data are presented as mean ± SD, n=3. **** (P<0.0001) using one-way ANOVA Dunnett's post hoc test. Data are all compared to live control.

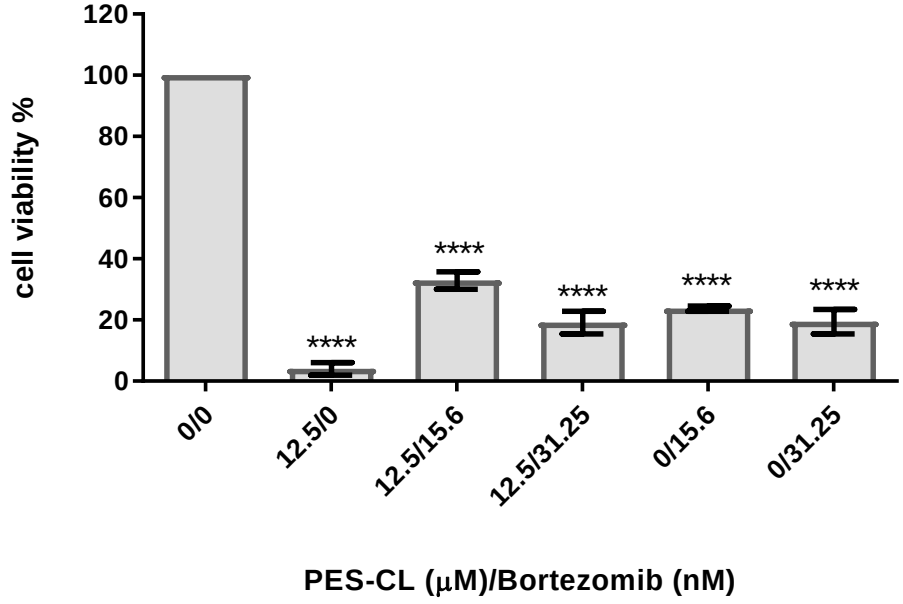


Figure 4.3.3.2. Effects on U937 cells (1x10^6 cells\ml) cell viability after 1 h Bortezomib treatment and subsequent 24 h treatment with PES-CL. After 24 h incubation, MTS assay was performed. Data are presented as mean ± SD, n=3. **** (P<0.0001) using one-way ANOVA Dunnett's post hoc test. Data are all compared to live control.

4.3.4. Synergy, additive and antagonism analysis on cell viability combined experiments

The data of all the MTS experiments were analysed using the Compusyn software to determine if the combination between HSPIs and Bortezomib may be synergistic, additive or antagonistic. Following the analysis, it emerged that all the combination used in these experiments were antagonistic; indeed, all the CI were found > 1, which is a clear sign of antagonism as described on section 2.3.9. The results were consistent regardless from the order of administration of the drugs and the cell line (Table 4.3.4.1.).

COMBINATION EXPERIMENT	**CELL LINE**	**COMBINATION INDEX** (for each combined concentration)	**RESULT**
PES-CL 1 h and Bortezomib 24 h	K562	12.5\15.6 = 1.69 12.5\31.2 = 1.99	Antagonism
Pifithrin-μ 1 h and Bortezomib 24 h	K562	12.5\15.6 = 2.37 12.5\31.2 = 2.35	Antagonism
Pifithrin-μ 1 h and Bortezomib 24 h	U937	12.5\15.6 = 1.84 12.5\31.2 = 2	Antagonism
Bortezomib 1 h and Pifithrin-μ 24 h	U937	12.5\15.6 = 9.53 12.5\31.2 = 3.77	Antagonism
PES-CL 1 h and Bortezomib 24 h	U937	12.5\15.6 = 1.52 12.5\31.2 = 1.81	Antagonism
Bortezomib 1 h and PES-CL 24 h	U937	12.5\15.6 = 5.57 12.5\31.2 = 2.14	Antagonism

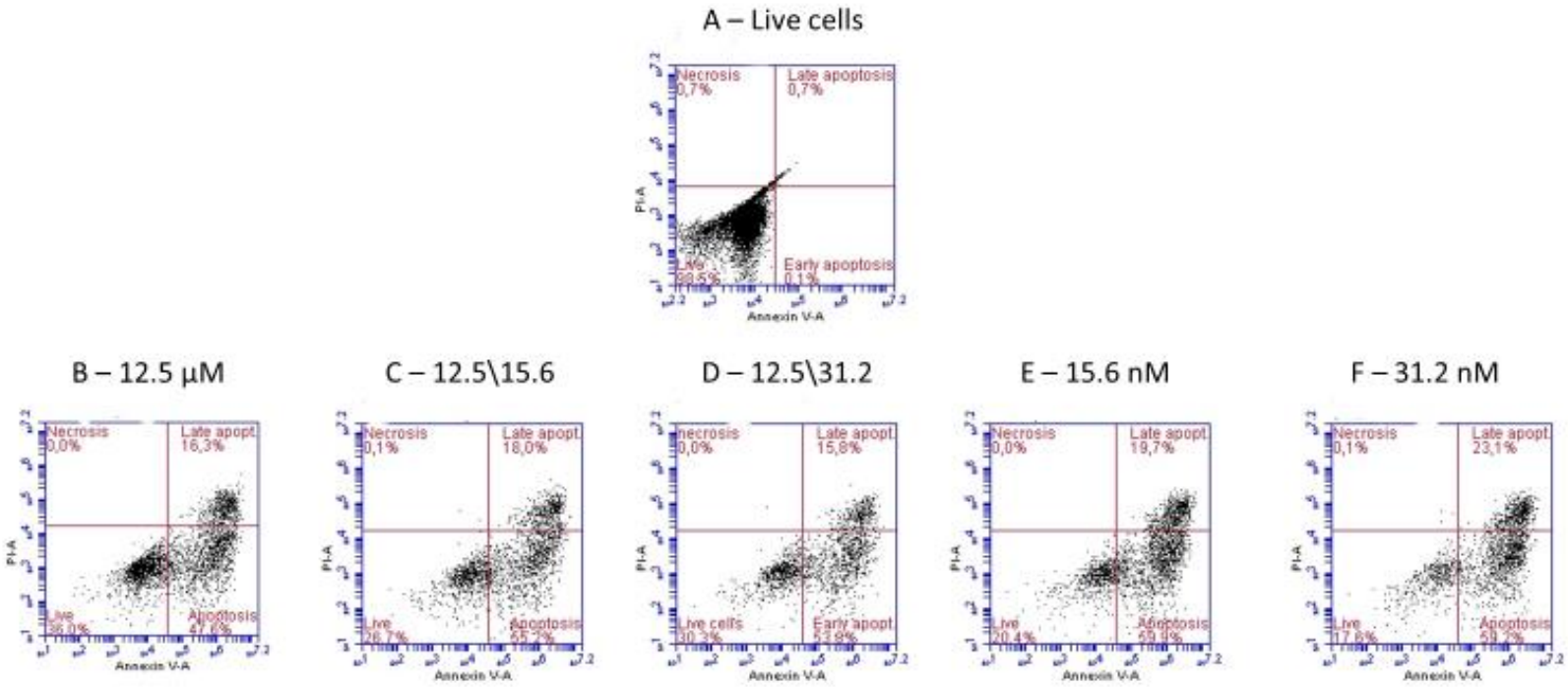
Table 4.3.4.1. Results of synergy, additive and antagonism analysis on cell viability combined experiments. The combination experiments all resulted with a CI > 1, therefore having an antagonistic effect. Importantly, the experiments are listed in the exact order as presented in the result section.

4.3.5. Cell death investigation through Annexin V\PI assay on the flow cytometer on K562 cells following Pifithrin-μ and Bortezomib combined therapy

To further understand the type of cell death induced by the treatment of Bortezomib and Pifithrin-μ both as single agents and combined on K562, the Annexin V\PI assay was performed. Similar to the previous chapter, the conditions of the experiment were the same of the MTS assay. In fact, following 1 hour of treatment with Bortezomib 15.6 nM and 31.2 nM, K562 cells were treated with 12.5 μM for 24 hours. The results are illustrated on Fig. 4.3.5.1. When Pifithrin-μ was added as single agent, it induced early apoptosis to 47.6 % of cells. A small percentage of cells died by late apoptosis, 16.3 %. The remaining 36 % of K562 cells where still live after 24 hours single agent treatment. Importantly, there was no sign of necrosis (B). When Pifithrin-μ was added following an hour treatment with Bortezomib at 15.6 nM, no necrosis was detected. Live cells percentage found was 26.7 %, whilst 55.2 % of K562 cells died by early apoptosis. Another percentage of cells, 18 %, died by late apoptosis (C). Also, when Pifithrin-μ was added to 31.2 nM, 53.8 % of cells died by early apoptosis; other 15.8 % died by late apoptosis instead. The cells which were not affected by the combined treatment were 30.3 %. No necrotic cells were found (D). With respect of Bortezomib concentrations as single agents, at 15.6 n M K562 cells died by early apoptosis (59.9 %). Late apoptosis levels were detected as 19.7 % and live cells as 20.4 %, confirming the effectiveness of the drug on K562 cells (E)When K562 cells were treated with 31.2 n M, similar apoptosis levels were detected (59.2 %). Only 17.6 % of cells were not affected by the treatment, whilst 23.1 % of the cells died by late apoptosis (F).

Here on Fig. 4.3.5.2 are represented the statistical data of the four quadrants of the previous data: live cells, early apoptosis, late apoptosis and necrosis. For each quadrant, all the single agent concentrations and the combined concentrations were compared to live control cells, in order to statistically evaluate the effectiveness of the treatments in terms of apoptosis. Live cells quadrant showed that there is a significant difference ($P<0.0001$) between live control and treated cells with 12.5 μM Pifithrin-μ. Similar statistical difference ($P<0.0001$) between live control and treated cells was found at 12.5\15.6 combination. Also, when cells are treated with 31.2 nM Bortezomib only, the difference between control and treated cells was calculated as ($P<0.0001$). The combination 12.5\31.2 when compared to live control cells resulted statistically different as ($P<0.0001$), whilst 15.6 nM single agent compared to live cells resulted different as ($P<0.0001$) (A). Early apoptosis quadrant was also analysed; the 12.5 μM treatment was compared to live cells control resulted significantly different as ($P<0.0001$). Same statistical difference was found on the combination 12.5\15.6 nM and when 15.6 nM was added as single agent, when they were compared to live cells control. Also, the combination 12.5\31.2 resulted different as ($P<0.0001$) in the comparison with live cells and when 31.2 n M Bortezomib was added as single agent, ($P<0.0001$) resulted as difference in the comparison with live cells (B). With respect of late apoptosis quadrant, 12.5 μM single agent data were compared to live cells control, resulting to have a ($P<0.01$) difference, whilst all the others combination resulted to be statistically different as ($P<0.05$) in comparison with live cells control. Only 31.2 n M was not considered statistically significant when compared to live cells (C). No necrosis was found, as mentioned above (D).

Fig. 4.3.5.1. Apoptosis and necrosis levels following 1 h treatment with Pifithrin-μ and 24 h subsequent treatment with Bortezomib on K562 cells. The analysis was performed following 24 hours treatment of combined and single agent drugs on K562 cells (1x10^{6}cells\ml). Respectively, A: Live cells, B: 12.5μM Pifithrin-μ, C: 12.5\15.6 combination, D: 12.5\31.2 combination, E: 15.6 nM Bortezomib, F: 31.2 nM Bortezomib. Data are representative of one replicate, as an example. Overall the samples were analysed in triplicates for each treatment and for the control.



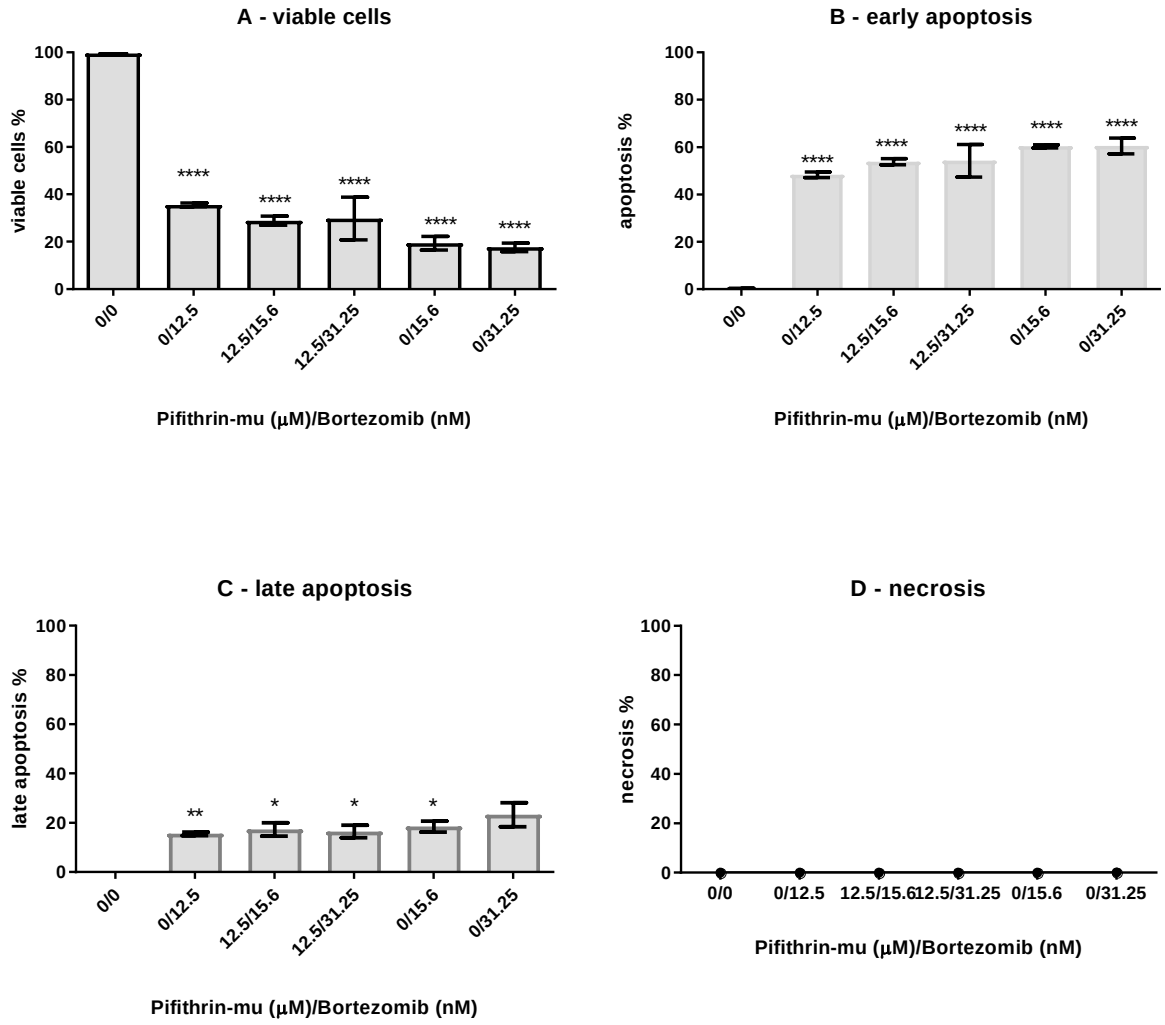


Fig. 4.3.5.2. Effects of combined treatment with Pifithrin-μ 1 h and Bortezomib 24 h on K562 cells on viable cells, early apoptosis, late apoptosis and necrosis. K562 cells ($1x10^6$ cells\ml) were treated with the combined therapy and, following 24 hours and Annexin V\PI assay, the cell conditions were plotted and analysed. A: viable cells levels, B: early apoptosis levels, C: late apoptosis, D: necrosis levels. Data are presented as mean ± SD, n=3. * (P<0.05), ** (P<0.01), **** (P<0.0001) using one-way ANOVA Dunnett's post hoc test. Data are all compared to live control.

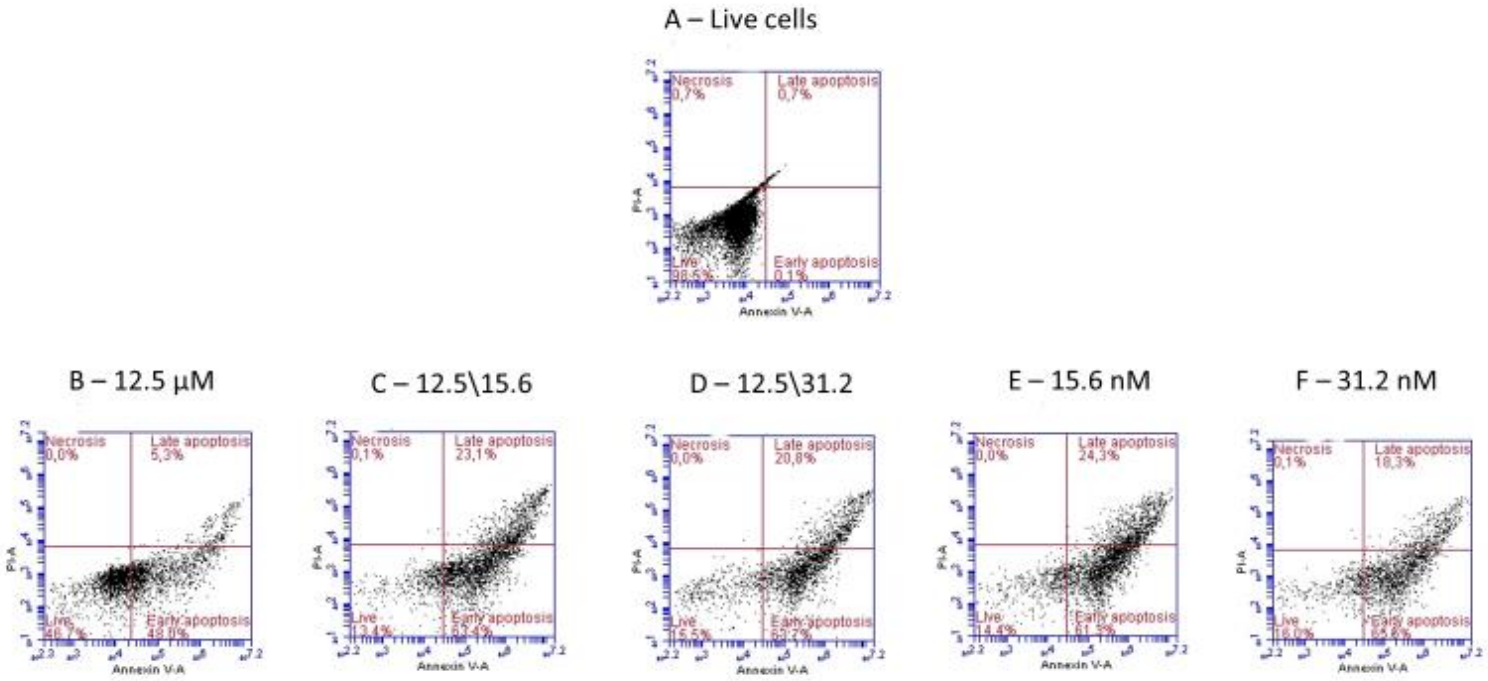
4.3.6. Cell death investigation through Annexin V\PI assay on the flow cytometer on K562 cells following PES-CL and Bortezomib combined therapy

The investigation on the type of cell death following a combination treatment was performed also with PES-CL and Bortezomib. Following 24 hours of treatment, whether PES-CL or Bortezomib was administered for an hour, Annexin V\PI assay was performed. No necrosis was detected for both experiments.

When 12.5 µM PES-CL was administered for 24 hours, 48 % of K562 cells died by early apoptosis, whilst 5.3 % of cells died by late apoptosis. The remaining cells were not affected by the single agent treatment (46.7 %), as shown in Fig. 4.3.5.1. (B). When Bortezomib was added to K562 cells, more cells died by early apoptosis than when they were treated with PES-CL as single agent. When 15.6 n M Bortezomib was added, 63.4 % of cells died by early apoptosis and 23.1 % as late apoptosis. The percentage of cells surviving the treatment was calculated as 13.4 % (C). When K562 cells were treated with an hour PES-CL and the subsequent 31.2 n M Bortezomib treatment, 63.7 % of cells died by early apoptosis and 15.5 % survived the treatment; 20.8 % of cells died by late apoptosis (D). 15.6 n M of Bortezomib, added as a single agent to K562 cells, resulted in 14.4 % of live cells and a small percentage of cells dying of late apoptosis, 24.3 %. The remaining 61.3 % died by early apoptosis (E). Also, when 31.2 n M of Bortezomib was added to K562 cells, only 16 % survived. Early apoptosis levels were detected as 65.6 %, whilst 18.3 % cells died by late apoptosis (F).

Fig. 4.3.6.2. showed the results of the statistical data of the four quadrants of the previous data: live cells, early apoptosis, late apoptosis and necrosis. For each quadrant, all the single agent concentrations and the combined concentrations were compared to live control cells, in order to statistically evaluate the effectiveness of the treatments in terms of apoptosis. With respect of live cells quadrant, 12.5 µM PES-CL was found statistically different as ($P<0.001$) when it was compared to live cells control; ($P<0.00001$) was also the statistical difference found in the comparison between 12.5\15.6 and live cells control. The remaining concentration, 12.5\31.2 combination, 15.6 nM Bortezomib single agent and 31.2 nM Bortezomib single agent, resulted statistically different to live cells control as ($P<0.0001$) difference (A). With respect of early apoptosis quadrant, 12.5 µM PES-CL resulted different as ($P<0.0001$) when compared to live cells control. Same difference was found at 12.5\31.25 combination in comparison with live cells control; at 15.6 nM Bortezomib single agent and at 12.5\15.6 combination the comparison between live cells and the treatments was calculated as ($P<0.0001$) statistical difference. The last concentration, 31.2 nM Bortezomib single agent, was found different as ($P<0.0001$) compared to live cells (B). Late apoptosis quadrant shown that 12.5 µM was not found significantly different in comparison with live cells control, whilst 12.5\15.6 combination was significantly different ($P<0.001$) compared to live cells control. The following combination treatment, 12.5\31.2, was found to be statistically different as ($P<0.01$) compared to live cells control. ($P<0.01$) was also the result of the comparison between live cells control and 31.2 nM Bortezomib. 15.6 nM Bortezomib single agent was not found significantly different (C). There is no necrosis, as shown on the previous picture (D).

Fig. 4.3.6.1. Apoptosis and necrosis levels following 1 h treatment with PES-CL and 24 h subsequent treatment with Bortezomib on K562 cells. The analysis was performed following 24 hours treatment of combined and single agent drugs on K562 cells (1x10^{6}cells\ml). Respectively, A: Live cells, B: 12.5µM Pifithrin-µ, C: 12.5\15.6 combination, D: 12.5\31.2 combination, E: 15.6 n M Bortezomib, F: 31.2 n M Bortezomib. Data are representative of one replicate, as an example. Overall the samples were analysed in triplicates for each treatment and for the control.



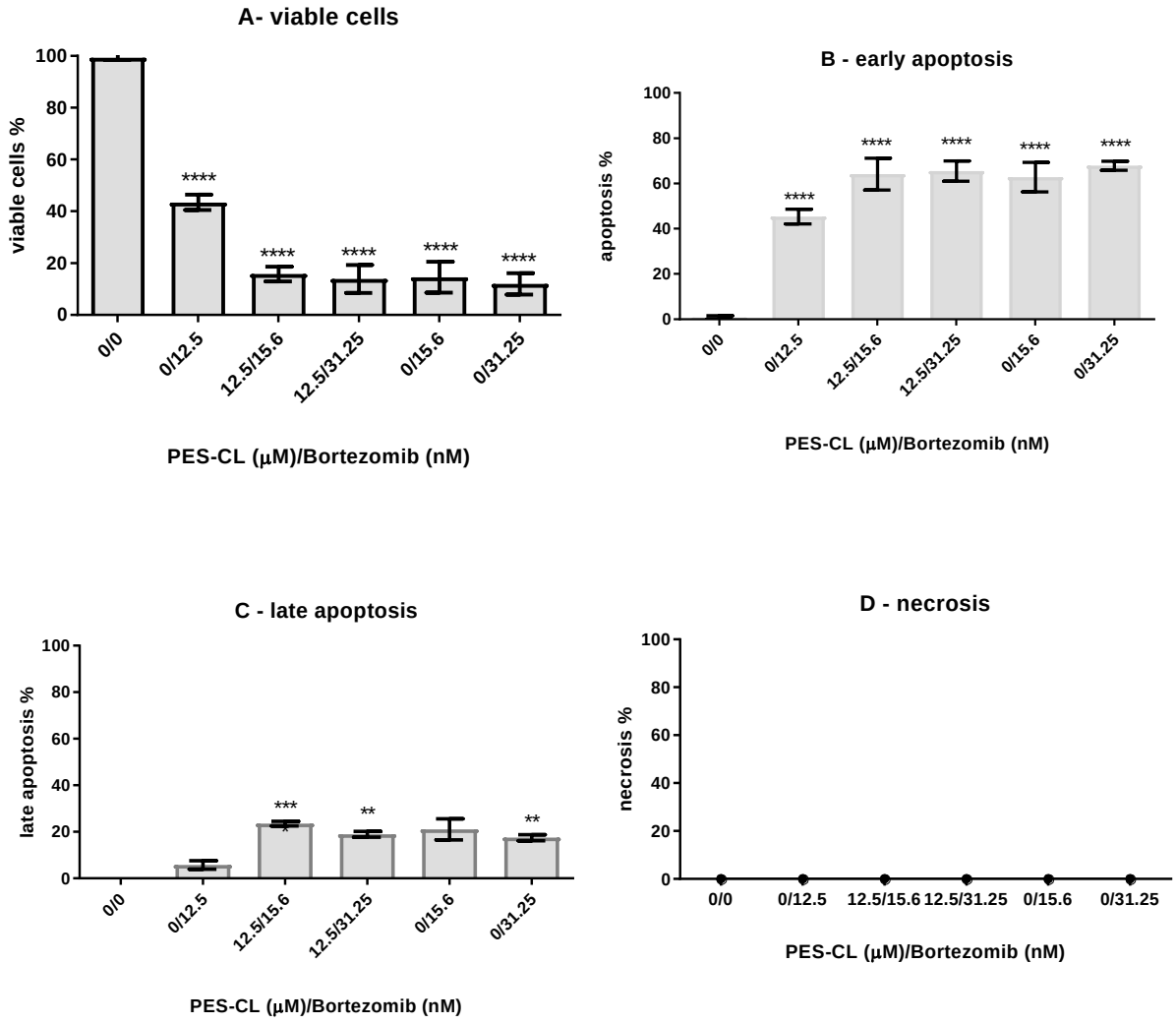
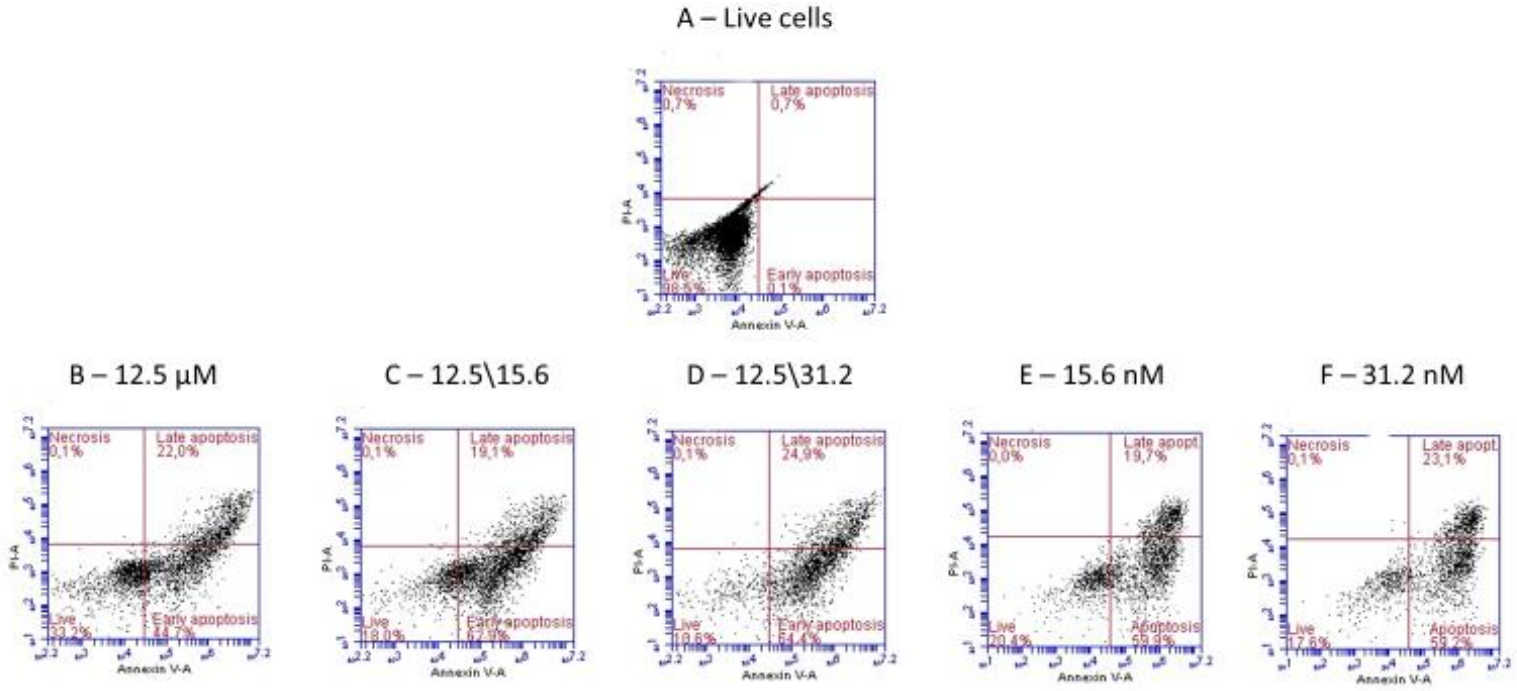


Fig. 4.3.6.2. Effects of combined treatment with PES-CL 1 h and Bortezomib 24 h on K562 cells on viable cells, early apoptosis, late apoptosis and necrosis. K562 cells ($1x10^6$ cells\ml) were treated with the combined therapy and, following 24 hours and Annexin V\PI assay, the cell conditions were plotted and analysed. A: viable cells levels, B: early apoptosis levels, C: late apoptosis, D: necrosis levels. Data are presented as mean ± SD, n=3. * (P<0.05), ** (P<0.01), *** (P<0.001), **** (P<0.0001) using one- way ANOVA Dunnett's post hoc test. Data are all compared to live control.

When Bortezomib was administered first, 12.5 µM PES-CL treatment following an hour of Bortezomib resulted in 44.7 % of dead cells by apoptosis and 22 %. The remaining cells are live, 33.2 % (Fig. 4.3.6.3. B). When PES-CL is added to 15.6 Bortezomib on K562 cells, only 18 % of cells were not affected by the treatment. Late apoptosis levels were detected as 19.1 %, whilst the majority of cells died of early apoptosis, 62.9 % (Fig. 4.3.6.3. C). PES-CL 12.5 µM was also added to K562 cells that were treated with 31.2 n M Bortezomib, showing 64.4 % early apoptosis and 24.9 % late apoptosis. 10.6 % of cells survived the treatment, as shown on Fig. 4.3.6.3. (D). Bortezomib has been administered for 24 hours also; at 15.6 nM 59.9 % of the cells died by early apoptosis and 19.7 % of late apoptosis. The remaining 20.4 % survived the treatment (Fig. 4.3.6.3. E). With respect of 31.2 n M, 59.2 % K562 cells died by early apoptosis and 23.1 % as late apoptosis; only 17.6 % of cells survived the treatment (Fig. 4.3.6.3. F). Necrosis has not been detected on any drug combinations.

Fig. 4.3.6.4. showed the results of the statistical data of the four quadrants of the previous data: live cells, early apoptosis, late apoptosis and necrosis. For each quadrant, all the single agent concentrations and the combined concentrations were compared to live control cells, in order to statistically evaluate the effectiveness of the treatments in terms of apoptosis. With respect of live cells quadrant, all the drugs concentrations, whether combined or single agents were found statistically different as ($P<0.0001$) when compared to live cells control (A). Early apoptosis levels quadrant showed that 12.5 µM was found statistically different as ($P<0.05$) in comparison with live cells control, whilst the combination treatments 12.5\15.6 and 12.5\31.2 were found statistically different as ($P<0.01$) to live cells control. Bortezomib single agent 15.6 nM, instead, was found different ($P<0.001$) to live cells control, whilst 31.2 nM Bortezomib single agent was found different as ($P<0.0001$) in comparison with live cells control (B). With respect of late apoptosis quadrant, 12.5 µM PES-CL resulted different ($P<0.01$) to live cells, whilst 31.2 nM Bortezomib was not found statistically significant. The remaining concentrations were instead found different as ($P<0.005$) in comparison with live cells control (C). Necrosis levels were not detected, as previously found (D).

Fig. 4.3.6.3. Apoptosis and necrosis levels following 1 h treatment with Bortezomib and 24 h subsequent treatment with PES-CL on K562 cells. The analysis was performed following 24 hours treatment of combined and single agent drugs on K562 cells (1x10^{6}cells\ml). Respectively, A: Live cells, B: 12.5µM Pifithrin-µ, C: 12.5\15.6 combination, D: 12.5\31.2 combination, E: 15.6 n M Bortezomib, F: 31.2 n M Bortezomib. Data are representative of one replicate, as an example. Overall the samples were analysed in triplicates for each treatment and for the control.



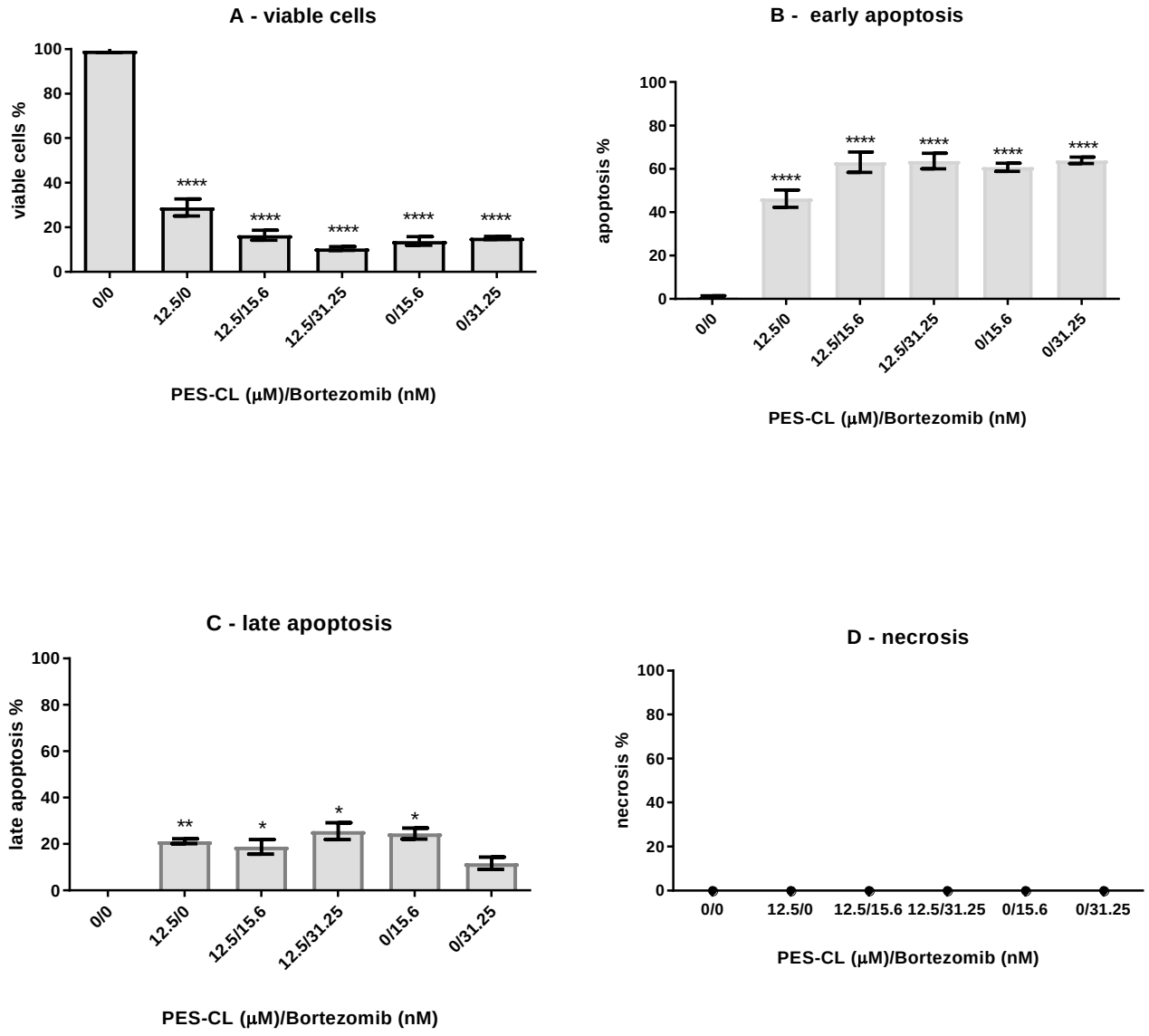


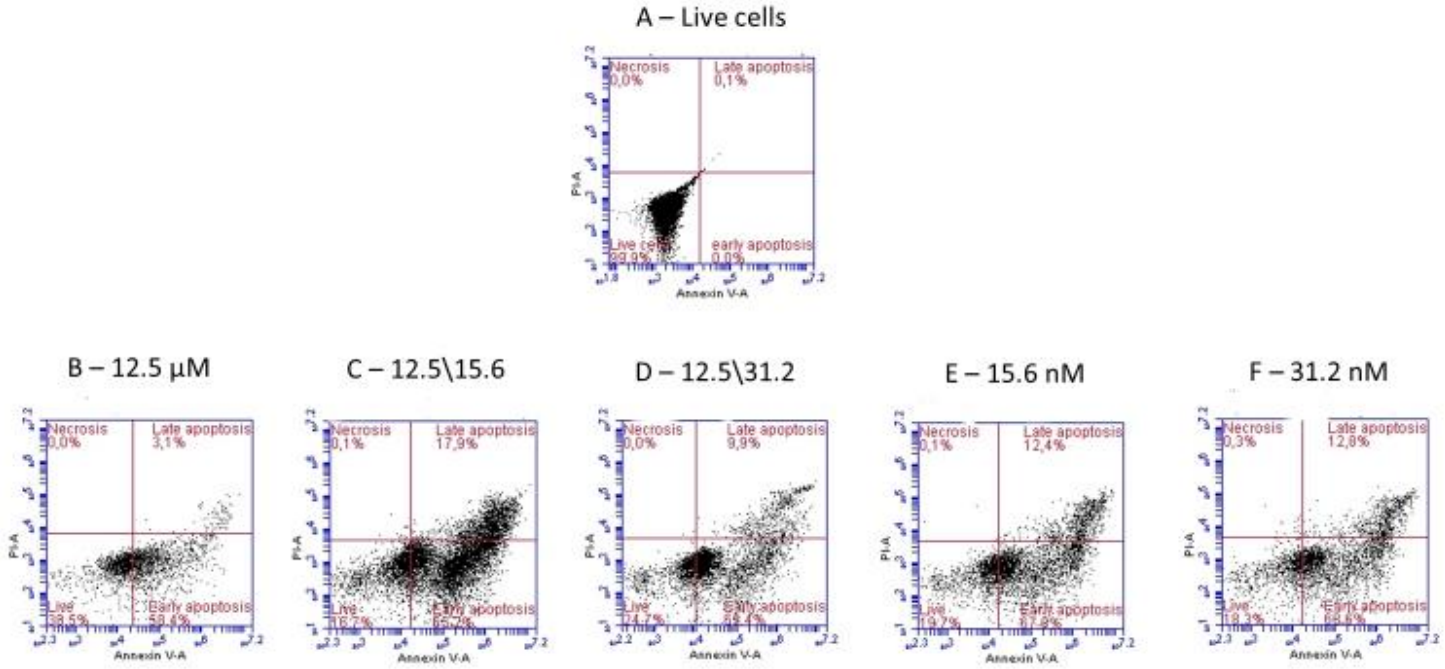
Fig. 4.3.6.4. Effects of combined treatment with PES-CL 1 h and Bortezomib 24 h on K562 cells on viable cells, early apoptosis, late apoptosis and necrosis. K562 cells ($1x10^6$ cells\ml) were treated with the combined therapy and, following 24 hours and Annexin V\PI assay, the cell conditions were plotted and analysed. A: viable cells levels, B: early apoptosis levels, C: late apoptosis, D: necrosis levels. Data are presented as mean ± SD, n=3. * (P<0.05), ** (P<0.01), *** (P<0.001), **** (P<0.0001) using one-way ANOVA Dunnett's post hoc test. Data are all compared to live control.

4.3.7. Cell death investigation through Annexin V\PI assay on the flow cytometer on U937 cells following PES-CL and Bortezomib combined therapy

U937 cells were treated also with PES-CL for an hour and Bortezomib was then added for 24 hours. At the end of the treatment, the type of cell death was investigated through the Annexin V\PI assay and the results are shown on Fig. 4.3.7.1. At 12.5 µM PES-CL, 58.4 % of the cells died by early apoptosis and only 3.1 % of late apoptosis; 38.5 % of U937 cells survived the treatment (B). At U937 cells previously treated with an hour of PES-CL was added 15.6 n M of Bortezomib; 65.7 % of cells died by early apoptosis and 16.7 % of them survived. The percentage of late apoptotic cells detected was 17.9 % (C). When 31.2 % was added after one hour, 65.4 % of U937 cells died by early apoptosis and 9.9 by late apoptosis. Only 24.7 % of the cells survived (D). When Bortezomib was added following one hour of PES-CL treatment, but as single agent, the apoptosis results were similar to the ones found when combined with PES-CL. Indeed, 19.7 % of the cells only survived at 15.6 n M, whilst 67.9 % of the cells died by early apoptosis and 12.4 % by late apoptosis (E). When the cells were treated with 31.2 n M of Bortezomib only, 68.6 % died by early apoptosis and 12.8 % by late apoptosis. Only 18.3 % of cells survived the treatment (F).

Fig. 4.3.7.2. showed the results of the statistical data of the four quadrants of the previous data: live cells, early apoptosis, late apoptosis and necrosis. For each quadrant, all the single agent concentrations and the combined concentrations were compared to live control cells, in order to statistically evaluate the effectiveness of the treatments in terms of apoptosis. With respect of live cells quadrant, 12.5 µM PES-CL was found different as ($P<0.0001$) to live cells control. The remaining combined concentrations, 12.5\15.6 and 12.5\31.2 and Bortezomib single agents 15.6 and 31.2 n M were found statistically different as ($P<0.0001$) in comparison to live cells control (A). Then, early apoptosis data were also analysed; PES-CL 12.5 µM resulted different as ($P<0.0001$) to live cells control; equally to what found with live cells quadrant, the remaining drugs combinations were calculated different as ($P<0.0001$), compared to live cells control (B). Late apoptosis statistical data was also analysed and it was found that PES-CL added for one hour first was calculated as not statistical different to live cells control. Instead, 12.5\15.6 and 12.5\31.2 combinations were found different ($P<0.05$) to live cells control; the two Bortezomib single agent treatments, 15.6 nM and 31 nM were found both statistically different as ($P<0.01$) to live cells control, as illustrated on (C). Consistently with previous results, no necrosis was analysed (D).

Fig. 4.3.7.1. Apoptosis and necrosis levels following 1 h treatment with PES-CL and 24 h subsequent treatment with Bortezomib on U937 cells. The analysis was performed following 24 hours treatment of combined and single agent drugs on U937 cells (1x10^{6}cells\ml). Respectively, A: Live cells, B: 12.5µM Pifithrin-µ, C: 12.5\15.6 combination, D: 12.5\31.2 combination, E: 15.6 n M Bortezomib, F: 31.2 n M Bortezomib. Data are representative of one replicate, as an example. Overall the samples were analysed in triplicates for each treatment and for the control.



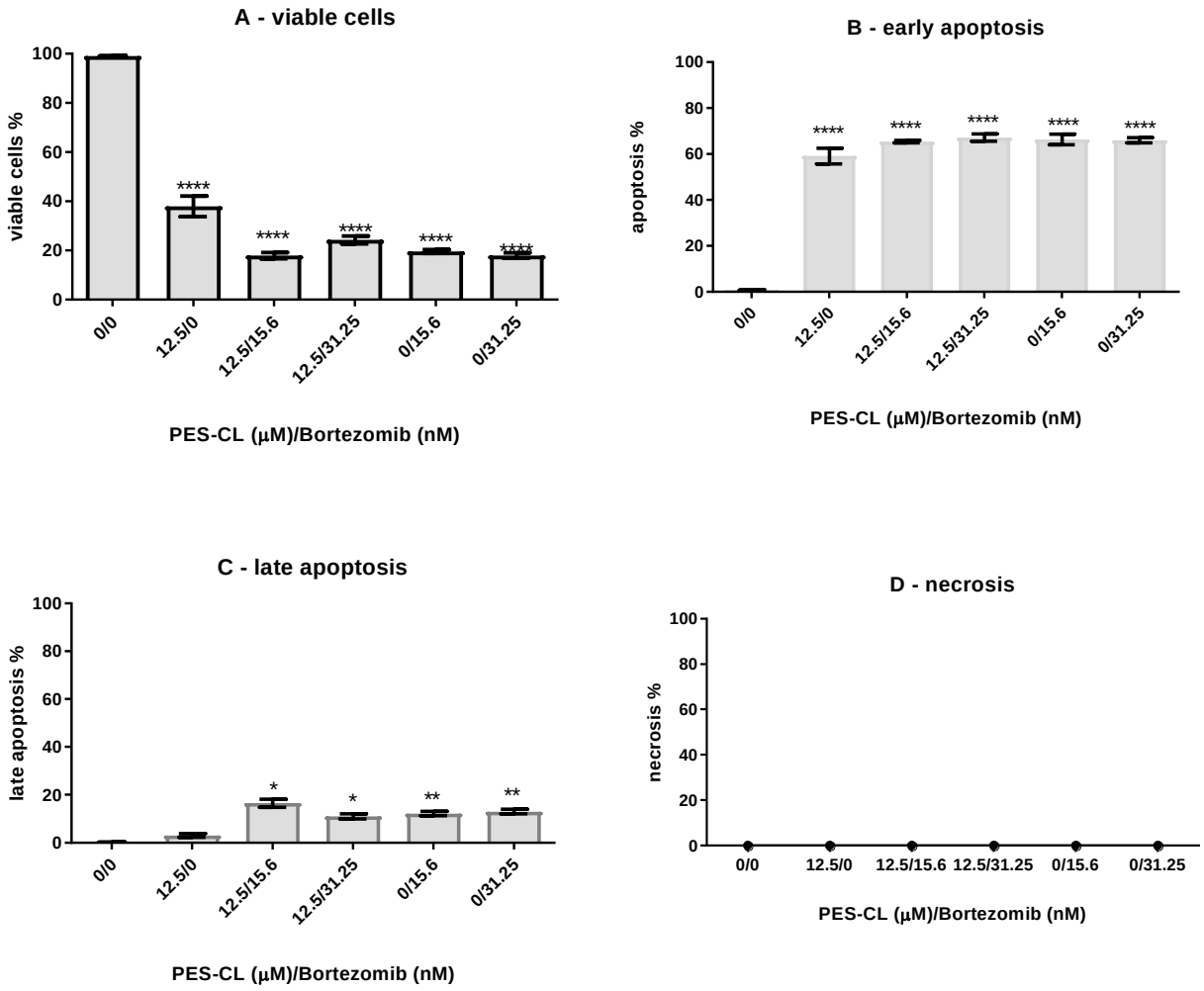
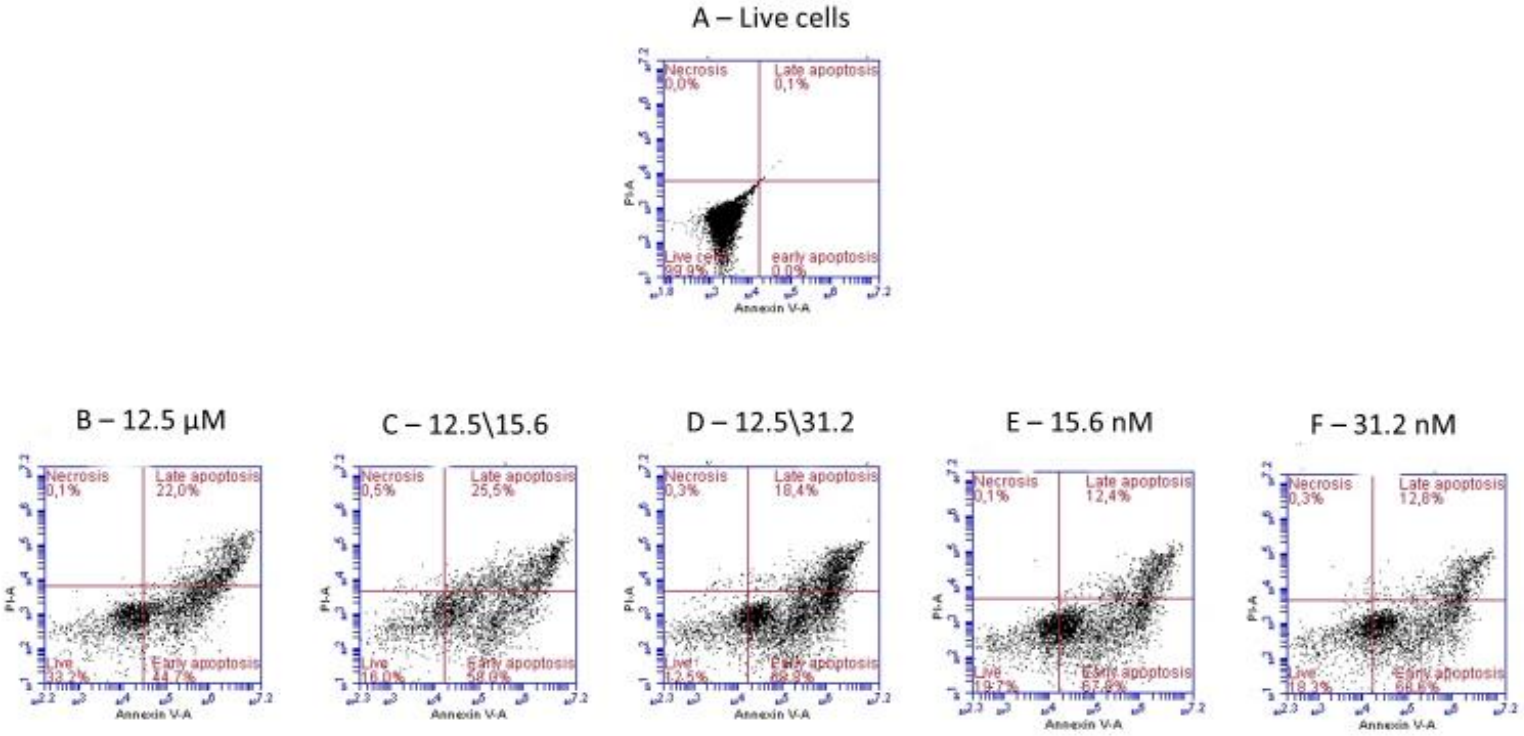


Fig. 4.3.7.2. Effects of combined treatment with PES-CL 1 h and Bortezomib 24 h on U937 cells on viable cells, early apoptosis, late apoptosis and necrosis. U937 cells ($1x10^6$ cells\ml) were treated with the combined therapy and, following 24 hours and Annexin V\PI assay, the cell conditions were plotted and analysed. A: viable cells levels, B: early apoptosis levels, C: late apoptosis, D: necrosis levels. Data are presented as mean ± SD, n=3. * (P<0.05), ** (P<0.01), **** (P<0.0001) using one-way ANOVA Dunnett's post hoc test. Data are all compared to live control.

When Bortezomib was added to U937 cells prior PES-CL treatment, the apoptosis levels resulted significant, as shown on Fig. 4.3.7.3. When Bortezomib was added as first drug at 15.6 n M concentration as a single agent, 67.9 % of the cells died by early apoptosis (E). Further, when 31.2 n M Bortezomib was added to U937 as single agent, 68.6 % of the cells died by early apoptosis and 12.8 % by late apoptosis. Live cells were detected as 18.3 % percentages (F). The 12.5\15.6 combination, where 12.5 µM PES-CL was added to cells an hour following Bortezomib treatment, showed that 58 % of the cells died by early apoptosis and 25.5 % of late apoptosis. Live cells percentage was 16 % (C). Also, when PES-CL was added to 31.2 n M, 69.8 % of the cells died by early apoptosis and 18.4 % by late apoptosis. The cells that did not survive the treatment were the 12.5 % (D). Finally, when PES-CL was administered as single agent, but one hour after Bortezomib treatment, U937 cells responded to the treatment less effectively than when PES-CL was combined. Indeed, 44.7 % of U937 cells died by early apoptosis and 22 % by late apoptosis; 33.2 % of the cells resulted still live following 24 hours treatment (B).

Fig. 4.3.7.4. showed the results of the statistical data of the four quadrants of the previous data: live cells, early apoptosis, late apoptosis and necrosis. For each quadrant, all the single agent concentrations and the combined concentrations were compared to live control cells, in order to statistically evaluate the effectiveness of the treatments in terms of apoptosis. With respect of live cells quadrant, all the concentrations, combined and single agents resulted as ($P<0.0001$) different to live cells control (A). Early apoptosis quadrant data showed that 12.5 µM resulted different as ($P<0.0001$) to live cells control; similar difference was found also on 12.5\31.2 combination and when 31.2 n M was administered as a single agent in a comparison to live cells control. At 12.5\15.6 and at 15.6 nM the difference found was also ($P<0.0001$), when they were both compared to live cells control (B). Late apoptosis statistical data were also plotted; at 12.5 µM single agent the difference between live cells and treatment was calculated as ($P<0.01$). At 12.5\15.6 the difference between live cells control and treatment was ($P<0.05$) and at 12.5\31.2 the difference between live cells control and treatment was ($P<0.0001$). The single agent concentrations of Bortezomib were not considered statistically significant, compared to live cells control (C). No necrosis was found (D).

Fig. 4.3.7.3. Apoptosis and necrosis levels following 1 h treatment with Bortezomib and 24 h subsequent treatment with PES-CL on U937 cells. The analysis was performed following 24 hours treatment of combined and single agent drugs on U937 cells (1x10^{6}cells\ml). Respectively, A: Live cells, B: 12.5µM Pifithrin-µ, C: 12.5\15.6 combination, D: 12.5\31.2 combination, E: 15.6 n M Bortezomib, F: 31.2 n M Bortezomib. Data are representative of one replicate, as an example. Overall the samples were analysed in triplicates for each treatment and for the control.



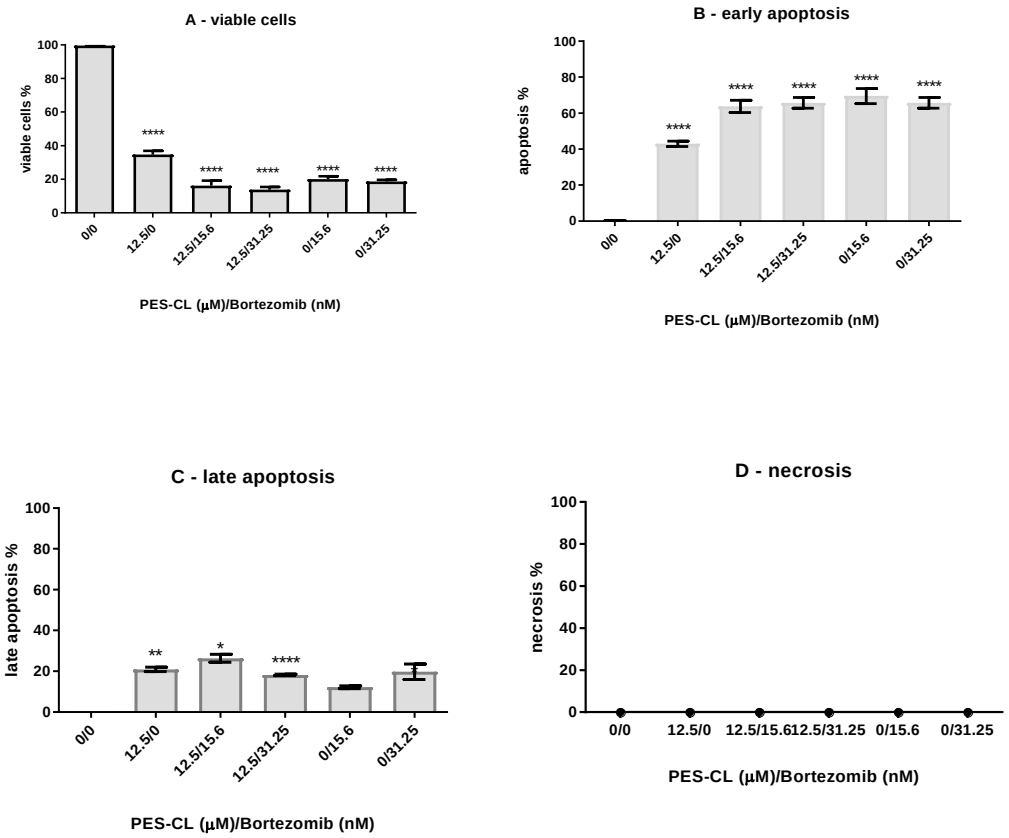


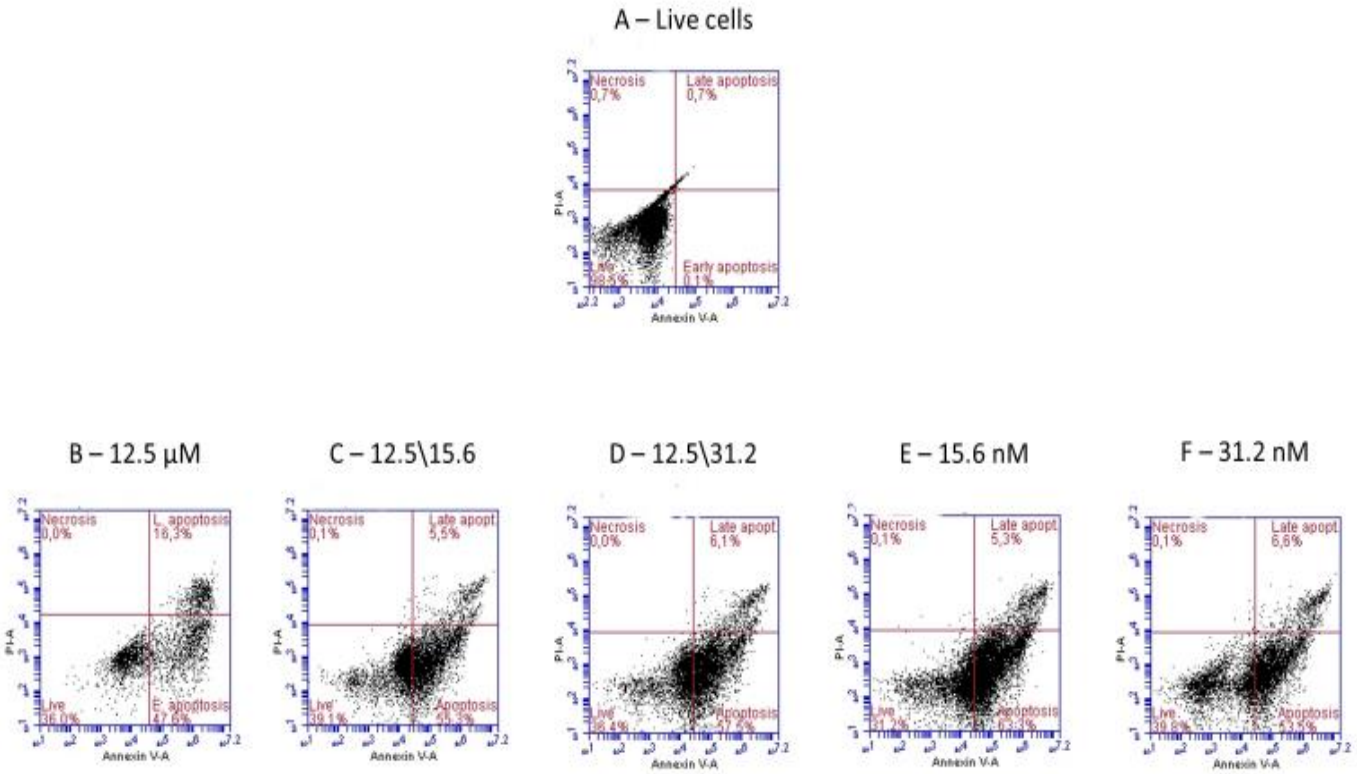
Fig. 4.3.7.4. Effects of combined treatment with Bortezomib 1 h and PES-CL 24 h on U937 cells on viable cells, early apoptosis, late apoptosis and necrosis. U937 cells ($1x10^6$ cells\ml) were treated with the combined therapy and, following 24 hours and Annexin V\PI assay, the cell conditions were plotted and analysed. A: viable cells levels, B: early apoptosis levels, C: late apoptosis, D: necrosis levels. Data are presented as mean ± SD, n=3. * (P<0.05), ** (P<0.01), *** (P<0.001), **** (P<0.0001) using one-way ANOVA Dunnett's post hoc test. Data are all compared to live control.

4.3.8. Cell death investigation through Annexin V\PI assay on the flow cytometer on U937 cells following Pifithrin-μ and Bortezomib combined therapy

Pifithrin and Bortezomib were tested as single agents and in subsequent combination on U937 as well. Annexin V\PI assay was performed to investigate the type of cell death and the results are shown on Fig. 4.3.8.1. When Pifithrin-μ was added first for an hour, at 12.5 μM 47.6 % of U937 cells died by early apoptosis and 16.3 % of late apoptosis; the percentage of live cells found was 36 % (B). When Bortezomib at 15.6 was added after an hour of Pifithrin-μ treatment, 55.3 % of the cells were detected as early apoptotic and 5.5 % as late apoptotic, 39.1 % of the cells were detected as live (C). 31.2 n M was also added to U937 cells treated with an hour Pifithrin-μ treatment, inducing early apoptosis to 57.5 % cells and 6.1 % late apoptosis; 36.4 % of the cells survived the treatment (D). When Bortezomib was added to U937 cells as single agent, at 15.6 n M the apoptotic cells that were detected were 63.3 % and the late apoptotic cells 5.3 %. The remaining 31.2 % were live cells (E). 31.2 n M was also added as single agent to U937, inducing early apoptosis to 53.5 % of cells and 6.6 % of late apoptosis cells. Live cells percentages detected was 39.8 % (F).

Fig. 4.3.8.2. showed the results of the statistical data of the four quadrants of the previous data: live cells, early apoptosis, late apoptosis and necrosis. For each quadrant, all the single agent concentrations and the combined concentrations were compared to live control cells, in order to statistically evaluate the effectiveness of the treatments in terms of apoptosis. With respect of live cells, 12.5 μM was found different as ($P<0.0001$) to live cells control; same difference was calculated for the combination 12.5\15.6 compared to live cells control. Also, 12.5\31.2 was found different ($P<0.0001$) to live cells control. Bortezomib single agent treatments, which are 15.6 nM and 31.2 nM, were found both different ($P<0.01$) to live cells control (A). Early apoptosis statistical data were also analysed; 12.5 μM was found different as ($P<0.0001$) to live cells control. Same statistical difference was found on 12.5\15.6 and on 12.5\31.2 was found different ($P<0.05$) in comparison to live cells control. Consistently with live cells statistical data, Bortezomib single agent concentrations, 15.6 nM and 31.2 nM were also found different as ($P<0.0001$) to live cells control (B). Late apoptosis statistical data were also analysed. Pifithrin-μ single agent 12.5 μM, was found different to live cells control as ($P<0.0001$). Interestingly, the remaining concentrations which are 12.5\15.6, 12.5\31.2 and 15.6 nM and 31.2 nM were all equally found different ($P<0.0001$) to live cells control (C). As previously mentioned in other paragraphs, no necrosis was found (D).

Fig. 4.3.8.1. Apoptosis and necrosis levels following 1 h treatment with Pifithrin-μ and 24 h subsequent treatment with Bortezomib on U937 cells. The analysis was performed following 24 hours treatment of combined and single agent drugs on U937 cells (1x10^6cells\ml). Respectively, A: Live cells, B: 12.5μM Pifithrin-μ, C: 12.5\15.6 combination, D: 12.5\31.2 combination, E: 15.6 nM Bortezomib, F: 31.2 nM Bortezomib. Data are representative of one replicate, as an example. Overall the samples were analysed in triplicates for each treatment and for the control.



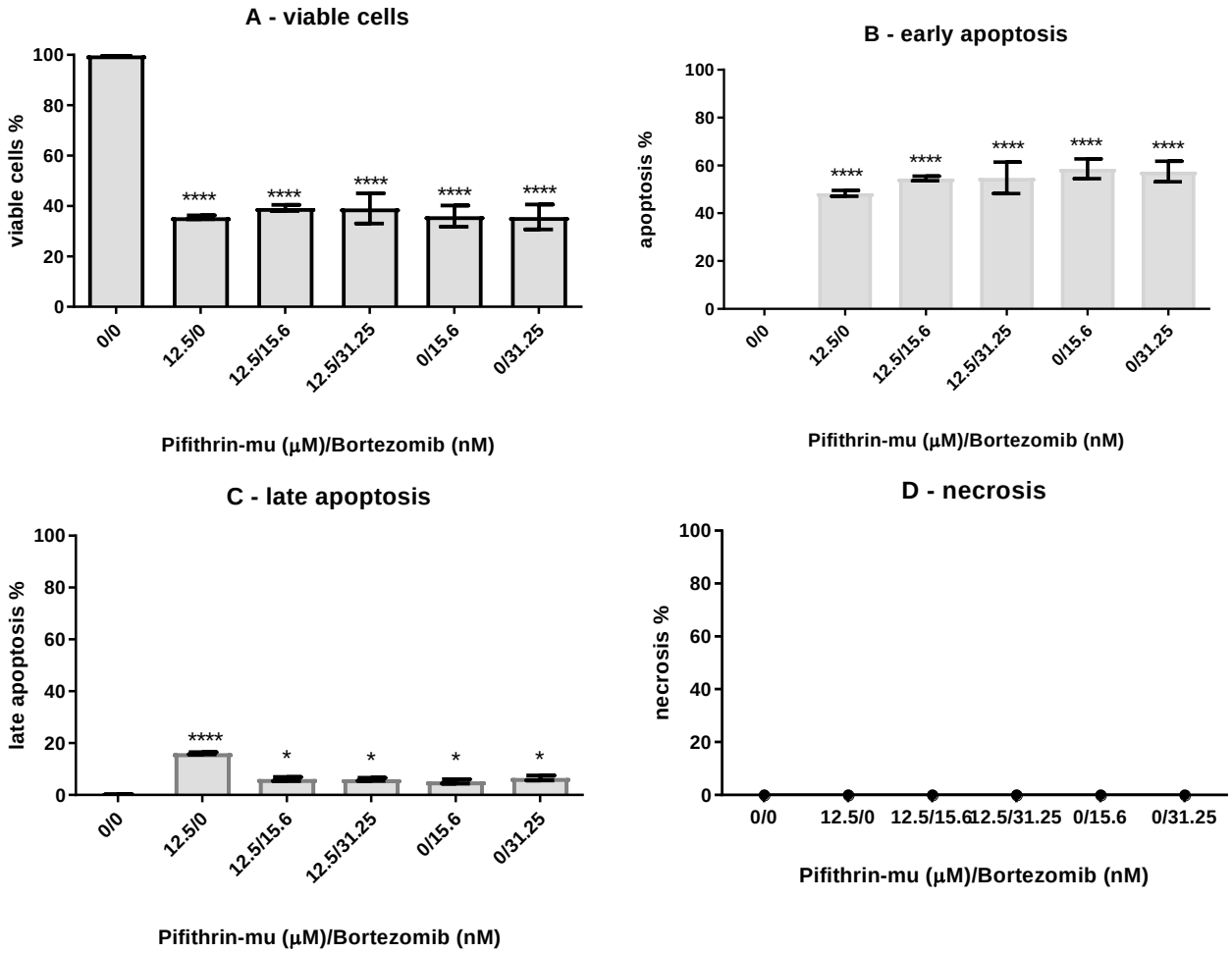
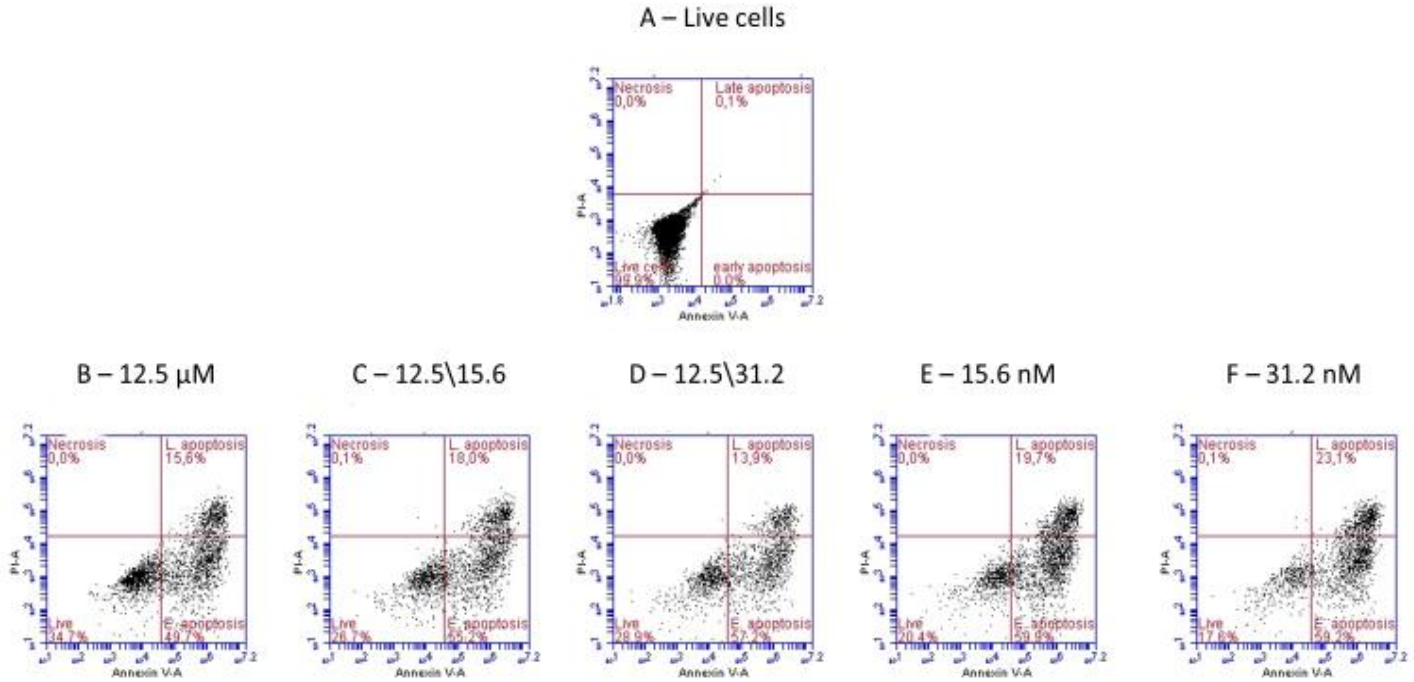


Fig. 4.3.8.2. Effects of combined treatment with Pifithrin-μ 1 h and Bortezomib 24 h on U937 cells on viable cells, early apoptosis, late apoptosis and necrosis. U937 cells ($1x10^6$ cells\ml) were treated with the combined therapy and, following 24 hours and Annexin V\PI assay, the cell conditions were plotted and analysed. A: viable cells levels, B: early apoptosis levels, C: late apoptosis, D: necrosis levels. Data are presented as mean ± SD, n=3. * (P<0.05), ** (P<0.01), **** (P<0.0001) using one-way ANOVA Dunnett's post hoc test. Data are all compared to live control.

When Bortezomib was added to U937 cells prior Pifithrin-μ treatment, 12.5 μM added an hour after Bortezomib treatment showed that 49.7 % of U937 cells died by early apoptosis and 15.6 % by late apoptosis. The remaining live cells detected were 34.7 % of the total (Fig. 4.3.8.3. B). When Pifithrin was added to 15.6 n M, 26.7 % of the cells did not respond to the treatment; 18 % of U937 were detected as late apoptotic and 55.2 % were found to be early apoptotic (Fig. 4.3.8.3. C). Also, 12.5 μM Pifithrin was added to 31.2 n M following an hour treatment; the combined treatment resulted in 57.2 % early apoptotic cells and 3.9 % of late apoptotic cells. The remaining 28.9 % cells were detected as live cells (Fig. 4.3.8.3. D). When Bortezomib was added first, but as a single agent at 15.6 n M, 59.9 % of U937 cells died by early apoptosis and 19.7 % as late apoptosis. The remaining 20.4 % cells was detected as live cells (Fig. 4.3.8.3. E). With respect of 31.2 n M single agent, only 17.6 % of U937 cells survived the 24 hours treatment, whilst 59.2 % died by early apoptosis and 23.1 % as late apoptosis (Fig. 4.3.8.3. F). No necrosis was found following Annexin V\PI assay throughout the different combinations of drugs.

Fig. 4.3.8.4. showed the results of the statistical data of the four quadrants of the previous data: live cells, early apoptosis, late apoptosis and necrosis. For each quadrant, all the single agent concentrations and the combined concentrations were compared to live control cells, in order to statistically evaluate the effectiveness of the treatments in terms of apoptosis. Live cells data showed that excluding 12.5\31.2 which was found to be different as ($P<0.0001$) to live cells control, the remaining concentrations 12.5\15.6, single agent Pifithrin-m 12.5 μM and 15.6 nM and 31.2 nM Bortezomib single agents were all found different as ($P<0.0001$) in comparison to live cells control (A). Early apoptosis statistical data showed that at 12.5 μM, 12.5\15.6, 12.5\31.2 and 31.2 n M the statistical difference between live cells control and treatment concentrations was calculated as ($P<0.0001$). Also, 15.6 n M was found to be different as ($P<0.001$) to live cells control (B). With respect of late apoptosis statistical data, 12.5 μM was found statistically different as ($P<0.0001$) compared to live cells control. When it was combined with 31.2 nM, the difference between live cells control and treatment was found to be ($P<0.05$). At 12.5\15.6 and at 15.6 nM and 31.2 nM, the difference between live cells control and treatment concentrations was found to be ($P<0.01$) (C). Consistently with previous experiments mentioned above, necrosis was not found (D).

Fig. 4.3.8.3. Apoptosis and necrosis levels following 1 h treatment with Bortezomib and 24 h subsequent treatment with Pifithrin-μ on U937 cells. The analysis was performed following 24 hours treatment of combined and single agent drugs on U937 cells (1x10^{6}cells\ml). Respectively, A: Live cells, B: 12.5μM Pifithrin-μ, C: 12.5\15.6 combination, D: 12.5\31.2 combination, E: 15.6 n M Bortezomib, F: 31.2 n M Bortezomib. Data are representative of one replicate, as an example. Overall the samples were analysed in triplicates for each treatment and for the control.



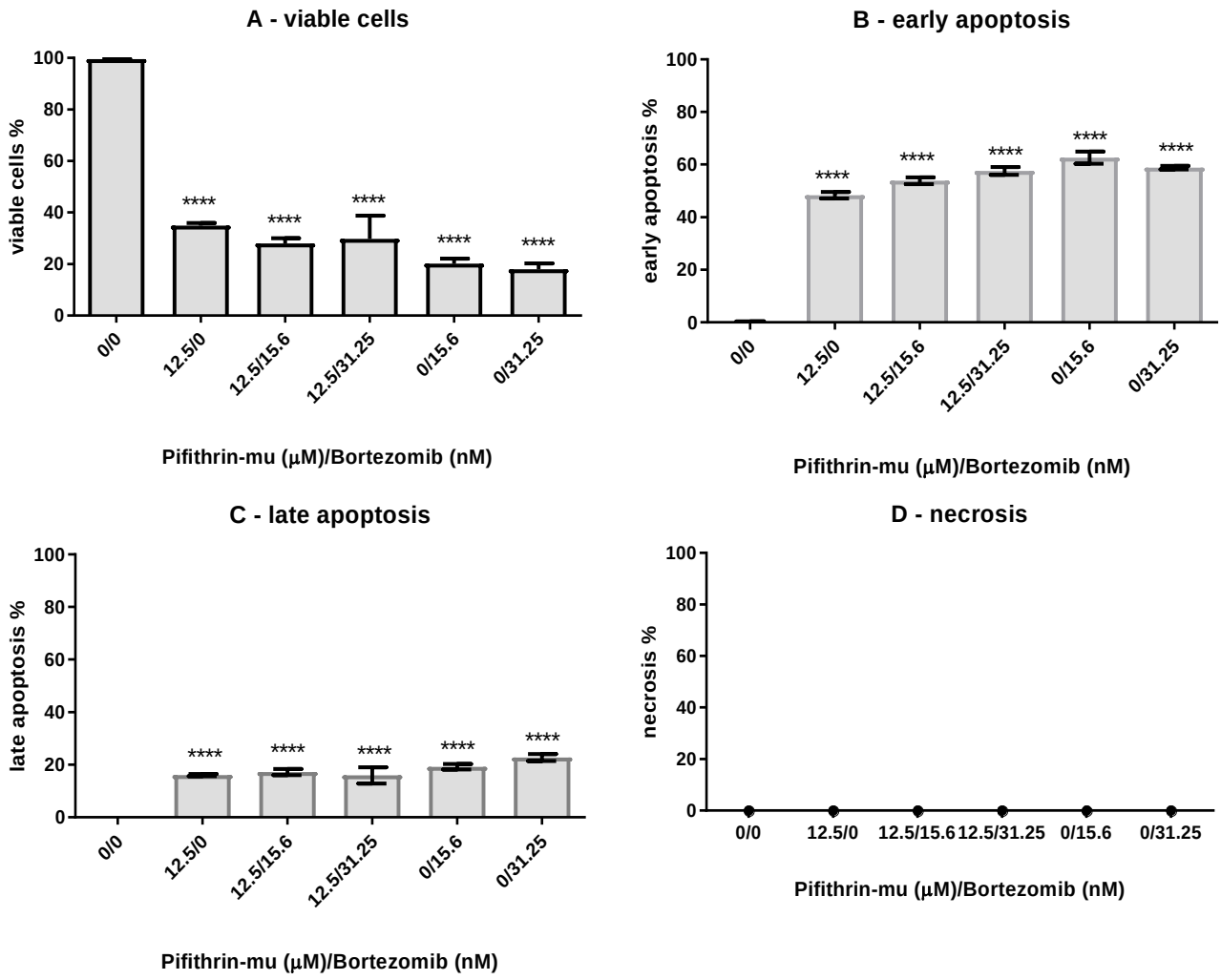


Fig. 4.3.8.4. Effects of combined treatment with Bortezomib 1 h and Pifithrin-μ 24 h on U937 cells on viable cells, early apoptosis, late apoptosis and necrosis. U937 cells ($1x10^6$ cells\ml) were treated with the combined therapy and, following 24 hours and Annexin V\PI assay, the cell conditions were plotted and analysed. A: viable cells levels, B: early apoptosis levels, C: late apoptosis, D: necrosis levels. Data are presented as mean ± SD, n=3. * (P<0.05), ** (P<0.01), *** (P<0.001), **** (P<0.0001) using one-way ANOVA Dunnett's post hoc test. Data are all compared to live control.

4.4. DISCUSSION

Heat shock proteins (HSPs) and the proteasome inhibitor Bortezomib have been demonstrated to affect cell viability and to induce apoptosis in a short time of administration, as mentioned in the previous chapter. However, it seemed interesting to investigate the potential use of these drugs in combination.

4.4.1. Effects of combined treatment with HSPIs and Bortezomib on cell viability of K562 and U937 cells

K562 cells responded to the combined treatment after one hour of Pifithrin-μ and subsequent treatment with Bortezomib for 24 hours. Particularly, the treatment with Pifithrin-μ as a single agent resulted in more dead cells than when Bortezomib was added as single agent or where the therapy was combined (Fig.4.3.1.1. and Fig.4.3.1.2.) Although the drugs combination resulted in a higher percentage of cells with affected metabolism compared to Bortezomib alone, these data may suggest that Pifithrin-μ may affect K562 cells independently from Bortezomib addiction. Perhaps, one hour of treatment with Pifithrin-μ is enough to kill leukemic cells and Bortezomib cannot bind to proteasomes. Contrary to these data, Pifithrin-α potentiated the effects of several drugs, such as doxorubicin, etoposide, and paclitaxel. Although the doses are not clearly stated and the drug is not the same, this is indicative of the antagonism of Pifithrin-μ, which has however a similar target and mechanism of action, and Bortezomib. Howewer, the effects were evaluated over a time of 96 hours, which is more than double to the time of treatment of this chapter. Also, the assay was performed on different cells, human colon carcinoma cell line HCT116 (Walton, M. I., Wilson, S. C., Hardcastle, I. R., Mirza, A. R., & Workman, P., 2005). Bortezomib administered first demonstrated not to enhance Pifithrin-μ activity, showing no difference when it was treated as single agent to when it was combined. These data may partially disagree to what found by Rodriguez, K. A., Osmulski, P. A., Pierce, A., Weintraub, S. T., Gaczynska, M., and Buffenstein, R., (2014) It seems that increasing concentration of Pifithrin-μ could affect the ability to convey resistance to Bortezomib treatment and to treatment with another proteasome inhibitor, MG-132 on naked mole rat cell lysates. The increasing concentrations of Pifithrin-μ used by this study go from 10 μM to 1000 μM, although it is not clearly stated how long Pifithrin-μ was administered for. This research suggested that due to a dose-dependent treatment with Pifithrin-μ and proteasome inhibitor, the proteasome activity of resistant cells was clearly and significantly inhibited. Also, immunoprecipitation analysis revealed a strong interaction between 26 S proteasome and anti-HSP70 antibody, suggesting a direct interaction between the two and an enhanced activity that fought the tendency to resist to proteasome inhibitors treatment. Cell viability via MTS assay was not measured. Although the cells and the techniques were different, these results seem to indicate that Pifithrin-μ and Bortezomib can interact and improve their respective mechanism of action; however, the results of this study strongly disagree with what demonstrated by Rodriguez, K. A., Osmulski, P. A., Pierce, A., Weintraub, S. T., Gaczynska, M., and Buffenstein, R, (2014).

On U937 cells, the combined therapy indicated that the treatment with Pifithrin-μ prior the addiction of Bortezomib seemed to be slightly more effective on cell viability compared to when Bortezomib was added first. These data suggest that when Pifithrin-μ is added first, U937 cells respond better to the combined treatment than when Bortezomib is administered as the first drug, particularly at 12.5\31.2. Indeed, considering the almost equal results when Pifithrin-μ and Bortezomib 31.2 nM were added as single agents and when these two concentrations were added together, it seems that Pifithrin-μ added first for an hour may be enough potent to kill leukemic cells, regardless from the enhancement given by 31.2 nM Bortezomib (Fig.4.3.2.1. and Fig.4.3.2.2.). This is not reflected by what demonstrated by Ocio E.M. *et al*. (2010) who suggested that when histone deacetylase inhibitor Panabinostat is added to multiple myeloma cells, there is a synergistic action with respect of cell cycle analysis. Interestingly, Panabinostat was also added to a mixture of Bortezomib and dexamethasone, therefore creating a triple treatment. On both cases, the cells were treated for 24 hours and Bortezomib dose was reduced from 15.6 nM to 5 nM and 3 nM (single agent treatment, double treatment and triple treatment, respectively) and analysed on flow cytometry. The results showed a significant reduction in the number of cells (%) in the S phase and G2 and M phases, indicating a synergic action of the drug combination with Bortezomib. Importantly, the data shown that when administered as single agent, there were more cells in those phases. Therefore, although contradicting the results of this chapter, it appeared that Bortezomib could enhance the activity on another drug. The antagonistic effect of HSPIs and Bortezomib found in this thesis is also supported by Kaiser *et al*. (2013), who treated U937 with sorafenib, cytarabine, SAHA (histone deacetylases inhibitors). The effects on cell viability were strongly reduced by the subsequent treatment with Pifithrin-μ if compared with SAHA treatment as single agent. Also, Pifithrin-μ was added simultaneously to sorafenib and cytarabine and after 48 hours incubation, cell viability was significantly reduced compared to sorafenib and cytarabine single agent treatment. Pifithrin-μ then showed to be effective in combination with other drugs which are not proteasome inhibitor on an AML cell line, confirming the antagonistic effect found on this thesis.

4.4.2. Effects of combined treatment with HSPIs and Bortezomib on apoptosis of K562 and U937 cells

Annexin V\PI experiments gave some results that confirmed what was suggested following the MTS assays; others, instead, disagreed. On K562, the combined therapy generally induced apoptosis to more cells than when both HSPIs were added as single agents, independently from the moment of administration, in agreement with (Ocio E.M. *et al*, 2010) which demonstrated that Bortezomib with a double or triple combination induced more apoptosis than when the two or three drugs were administered alone. This is particularly notable on the PES-CL – Bortezomib experiments (Fig.4.3.5.1. and Fig.4.3.5.3.). When PES-CL was combined with Bortezomib, the percentage of the cells dead by early apoptosis was improved than when it was single agent concentration, 63 % on both combinations. In agreement with this, Yu C. *et al*, (2006) demonstrated that Sorafenib and Bortezomib in combination induced more apoptosis than when they were added to K562 cells as single agents for 48 hours. Although the time of treatment was different and the dose of Bortezomib was much lower (6 nM), the results of this study could be an indication of a potential use of PES-CL with Bortezomib to induce significant apoptosis on K562 cells. This partially contradicted what has been demonstrated on the MTS assay, where the combination 12.5\15.6 resulted in an antagonist effect between the two drugs. However, it is important to state that the two different combinations did not result significantly different when compared between them and the two drugs as single

agents, not supporting the conclusion of synergistic effect. The antagonistic effect found in this thesis is not in agreement with what found by Duechler, M., Linke, A., Cebula, B., Shehata, M., Schwarzmeier, J. D., Robak, T. and Smolewski, P., (2005) who proved that subsequent administration of cladribine or fludarabine with Bortezomib induced significant apoptosis to CLL cells. In fact, low doses of these drugs, such as 2 nM Bortezomib for a longer treatment of 48 hours showed to be synergistic with other agents.

PES-CL and Bortezomib combinations on U937 confirmed what found on K562; indeed, PES-CL as a single agent was the least effective in terms of early apoptosis. The combined therapy both induced 65 % of early apoptosis, interestingly. This is partially supported by what demonstrated by Lum, M. A., Balaburski, G. M., Murphy, M. E., Black, A. R., & Black, J. D. (2013); indeed PES-CL protects PKC protein from proteasome degradation on rat intestinal crypt-like cells; although this thesis did not measure proteasome activity, it remains clear that PES-CL as a single agent could have a stronger effect than in combination and it is also confirmed to be more potent than Pifithrin-μ. Bortezomib single agent induced slightly higher results, confirming the most effectiveness within the whole range of concentration and, also, not confirming what found on MTS assay (Fig.4.3.6.1.). When Bortezomib was added first, the antagonistic effect was perfectly notable on 12.5\15.6 combination; this resulted in a lower level of apoptosis, on the contrary to the remaining single and combined treatment. Interestingly, the combination between the two lowest doses, 12.5\15.6, suggested that this could be the only combination where there is not a predominant drug, but the two drugs are competitive for the role of restoring proteasome activity (Fig.4.3.6.3.). This is also confirmed by another study; Bortezomib did not seem to induce apoptosis to colon cancer cell lines in combination with Nutlin following 24 hours treatment, according to what found on Annexin V\PI by Lee, D. M., *et al*. (2017). However, this study failed to show accurate graphs which may figuratively state that Bortezomib cannot induce apoptosis in combination with Nutlin. Further studies are needed to fully understand the potential combination between HSPIs and proteasome inhibitors in leukaemia.

CHAPTER 5: DOES HSP70 INHIBITION CAUSED BY COMBINED AND SINGLE TREATMENT OF HSPIs AND BORTEZOMIB AFFECT BCL-2 EXPRESSION ON LEUKEMIC CELL LINES?

5.1. INTRODUCTION

This chapter investigates the effects of combined therapy with HSPIs such as Pifithrin-μ and PES-CL in combination with the proteasome inhibitor Bortezomib and as single agents on HSP72 inhibition; it also investigates the effect of HSP72 inhibition on Bcl-2 expression, attempting to indicate which type of apoptosis occur following HSPIs and Bortezomib treatment on leukemic cell lines.

Due to its role in the protein folding activity, HSP72 plays an important role in apoptosis. Its activity seemed to be linked to both caspase dependent apoptosis and independent from caspases; the relation between HSP72 and Bcl-2 is still unclear, as the different in literature proves. As mentioned in the section 1.14, the C-terminal site can be compared to a lid; it seems that this site could be involved in the downstream regulation of caspase-3. A study by (Jaattela M., *et al*., 1998) demonstrated that HSP72 could inhibit the apoptotic cascade downstream of cytochrome-c release and caspase-3 activation. It has been demonstrated that it could inhibit mitochondrial pathway by inhibiting Apaf-1 and the consequential caspase signalling cascade, confirmed by the inhibition of Jnk pathway (Gabai V.L., *et al*., 2002). HSP72 has been demonstrated to inhibit the traslocation of apoptosis inducing factor (AIF) from mitochondria to the nucleus where it seems to induce the caspase-independent cell death on BCR-ABL cells; also, the over-expression of HSP72 is associated with increased levels of Bcl-2, which is paramount in the mitochondrial apoptosis pathway due to its blocking function to the release of cytochrome c and AIF in the cytosol (Wang F. *et al*, 2005 and Mayer, M. P. and Bukau B., 2005). HSP72 also binds to IκB disrupting the function of the kinase of IκB and blocking NF-κB pathway by degrading p65 and leading to DNA fragmentation independently from the caspase cascade signalling (Tanaka T., *et al*., 2014). HSP70 inhibition and the restoring of normal proteasome activity resulted particularly significant in the treatment of neuromuscular disorder congenital myasthenic syndrome; a genetic mutation in choline acetyltransferase activity seems to be the main cause of this syndrome. It has been found that HSP70 inhibitors reduced the choline acetyltransferase activity thus enhanced proteasome activity. However, HSP72 inhibition role is not completely clear in the apoptosis process with respect of this syndrome (Morey, T. M., *et al*., 2017). Another proof of the controversial role between HSP72 and apoptosis was suggested by (Leu, J. I.-J., *et al*., 2009) who have demonstrated that the inhibition of HSP70 prevents p53 to accumulate in the mitochondria, therefore suggesting that the mitochondrial apoptosis pathway is not triggered following Pifithrin-μ action. HSP72 inhibition showed to alter the lysosome function, leading to a dysfunctional autophagy, and to a different type of cell death than caspase dependant. However, other studies are not in agreement with this theory, suggesting that HSP72 did not inhibit the activity of caspase-3 in vitro, even in the presence of ATP and therefore the clear involvement of the C-terminal site mentioned previously. In fact, it seems that HSP72 could inhibit also independent caspase apoptosis, in addition to the caspase dependent action, confirming HSP72 involvement in both the type of apoptosis (Mosser D.D., *et al*., 1997 and Creagh E.M., *et al*., 2000). Also, another group of researchers suggested that HSP72 seemed to inhibit caspase-independent pathway by

inhibiting the apoptosis-inducing factor, escaping DNA fragmentation and caspase 8 involvement (Guo F. *et al*, 2005).

High levels of Bcl-2 have been found on 276 patients with Chronic Lymphocytic Leukaemia (CLL), according to a study by (Majid A., *et al*., 2008), which also urged to propose a mechanism which can help understanding the reasons of such overexpression. Patients with CLL lack Bcl-2 translocation in the chromosomes; this genetic mutation may be explained by loss of microRNA (miRNA) expression or expression of a protein called nucleolin, which plays a pivotal role in the synthesis and maturation of ribosomes. However, the reasons for Bcl-2 overexpression still remain unclear and need further studies. With respect to CML, high levels of Bcl-2 may be able to explain cell survival and resistance to treatment in the chronic phase of CML. Overexpressed protein was found on CML stem cells, which failed to be responsive to treatment of tyrosine kinase inhibitors, perhaps the paramount treatment for any CML patient. A potential inhibition of Bcl-2 combined with tyrosine kinase inhibitor treatment showed encouraging results, suggesting a potential role as a new combination therapy for CML patients (Carter B.Z. *et al*, 2017). Furthermore, a correlation between Bcl-2 polymorphism and CML has been proposed; Bcl-2 have two isoforms which are Bcl-2-α (26 kDa) and Bcl-2-β (22 kDa). The first one is associated with anti-apoptotic activity, due to its hydrophobic transmembrane domain bound to the mitochondrial membrane. The presence of one of these two isoforms could influence the balance between apoptotic and anti-apoptotic signals; it has been suggested that these polymorphisms may enhance the chances to develop CML and therefore influence the treatment of these patients (Guillem V. *et al*, 2015). Bcl-2 over-expression may play an even more important role on AML; in fact, it has recently emerged that high Bcl-2 levels are an important characteristic of quiescent stem cells, which are resistant to normal chemo-therapy (Lagadinou E.D. *et al*, 2013). Also, inhibition of Bcl-2 with Venetoclax on such cells induced apoptosis (Pullarkat V.A. and Newman E.M., 2016). Therefore, it seemed interesting to evaluate the inhibition of Bcl-2 on U937 cells, miming what could happen on an AML patient resistant to normal chemo-therapy.

The treatment of CML and AML could have as potential targets the proteasome and HSP70, as it has been demonstrated in the previous chapters. The 26S proteasome degrades various proteins critical to cancer cell survival, such as cyclins, tumour suppressors, BCL-2, and cyclin-dependent kinase inhibitors. Inhibition of these degradations sensitizes cells to apoptosis. Bortezomib upregulates the activity HSP70 proteins, together with proteins such as Noxa, Bcl-2 and Mcl-1 which are central in the apoptosis pathways. Due to the upregulation activities, Bortezomib is shown to trigger both caspase dependent and independent apoptosis (Selimovic D. *et al*, 2013). Bcl-2 and the proteasome are strongly connected; posttranslational modification of Bcl-2, such as dephosphorilation followed by stimuli by Tumour Necrosis Factor alpha (TNFα), induces ubiquitin-dependent degradation of the anti-apoptotic protein Bcl-2 (Breitschopf K., *et al*., 2000). Bortezomib, being a proteasome inhibitor, could potentially facilitate the degradation of Bcl-2 protein, restoring the balance between pro-apoptotic and anti-apoptotic proteins. However, the treatment with Bortezomib seemed to give opposite and conflictual results. A study by (Perez-Galan P. *et al*, 2006) have demonstrated that a conformational change might occur on Bax and Bak due to Bortezomib administration on patients with mantle cell lymphoma. Also, Bortezomib seemed to induce upregulation of NOXA, clear sign of apoptosis regulated by mitochondrial pathway. Bortezomib also seemed to upregulate MCL-1 which is a protein that interacts with pro-apoptotic proteins Bax and Bak inhibiting them. Following treatment with Bortezomib restored apoptosis signals; this was obtained also with combined

therapy with fludarabine or small interfering RNA on melanoma cells (Qin J.Z., *et al*., 2006). Bortezomib seems to induce apoptosis in a caspase independent fashion, involving caspase 8 instead. This suggests that Bortezomib could induce apoptosis also on diseases or patients where Bcl-2 is not inhibited or it is less sensitive to Bcl-2. Interestingly a study by (Chauhan D. *et al*, 2005), demonstrated that the combined therapy between Bortezomib and another proteasome inhibitor called NPI-0052 blocked proteasome activity in multiple myeloma cells. Further, it has been suggested that caspase 8 and caspase 9 may be responsible for the cell death, data which were confirmed when caspase 8 and caspase 9 were inhibited, resulting in a reduced death cell ratio. It was also concluded that Bortezomib can induce apoptosis via mitochondrial permeabilization, therefore somehow inhibiting Bcl-2, whilst the other proteasome inhibitor failed to do so (Chauhan D. *et al*, 2005).

Pifithrin-μ has been only recently considered as an HSP70 inhibitor; originally it was indeed developed as a p53 inhibitor. Specifically, Pifithrin-μ inhibits the translocation to the mitochondria of p53 without affecting the posttranscriptional mechanism. Bcl-2 therefore, it is regulated by p53 activity; it has been suggested that Pifithrin-μ is able to rescue DoHH2 and MCA cells from cell death in combination with Nutlin, a small molecule that activates p53 pathway. The results suggested that Pifithrin-μ may induce apoptosis in Bcl-2 and p53 independent fashion and further confirmed Bcl-2 role in cell survival (Drakos E. *et al*, 2010). The potential role in CML and AML treatment of HSPIs and Bortezomib would benefice from further studies with respect of apoptosis cascade signalling, particularly with respect of the relation between HSP72 and Bcl2. A potential answer to these questions may be given by the results of this study, whose aims are list in the following section.

The aims of this chapter are:

- To investigate the effects of combined and single agent therapy of HSPIs and Bortezomib on HSP72 inhibition on K562 cells.
- To investigate the effects of combined and single agent therapy of HSPIs and Bortezomib on HSP72 inhibition on U937 cells.
- To investigate the effects of combined treatment and single agent therapy of HSPIs and Bortezomib on Bcl-2 levels on U937 cells.

5.2. METHODS.

5.2.1 Cell culture

K562 and U937 cell lines were used for these set of experiments. The cells were cultured and subdivided as described in Chapter 2.3.1. Both cell lines were treated at a $1x10^6$ cells\ml concentration.

5.2.2. Cells treatment for flow cytometry assays

K562 and U937 cells were plated and treated as described on Chapter 2.3.5.3.

5.2.3. HSP72 expression on flow cytometry

The assay was performed as described on Chapter 2.3.7.2. In this section is important to highlight how the analysis of the data was performed and how the results are visualized on the results section. Importantly, the assay and the HSP70 FITC measured HSP72 inhibition; HSP72 belongs to the HSP70 family and it is overproduced in case of cellular stress (M. P. Mayer and B. Bukau, 2005). The FITC HSP70 antibody (Stressmarq) used specifically binds to the stress-inducible form HSP70, which is represented by HSP72. To measure HSP72 expression, the treated cells were either probed with HSP70 antibody, or were not probed to have a negative control for each treatment. A probed live untreated cells was also used to set a threshold: high signal intensity caused the shift to the right of such threshold. This happened on treated cells, for example. On the contrary, control with no antibody did not show a high intensity and there was no shift in respect of the threshold. Below there is an example of two different histograms which represent a control of treated cells with no FITC HSP70 antibody and treated cells with FITC HSP70 antibody. Purely as an example, 12.5 µM single agent on K562 cells was chosen.

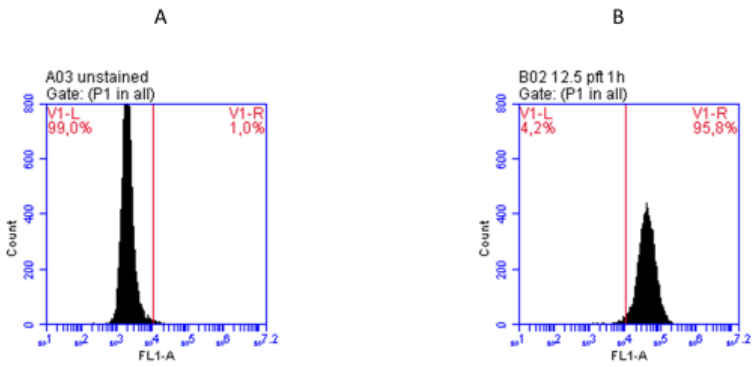


Fig 5.2.3.1. Example of histograms of HSP70 inhibition on flow cytometer: the first histogram represents live cells with no HSP70 FITC antibody; there is no signal and the cells do not pass the set threshold (A). On the contrary, the second histogram represents treated cells with FITC HSP70 antibody; the population shifted to the right of the red line because of high signal intensity, indicating the HSP72 expression (B).

5.2.4. Bcl-2 expression on flow cytometry

The assay was performed as described on Chapter 2.3.7.2. In this section is important to highlight how the analysis of the data was performed and how the results are visualized on the results section. U937 cells were plated on a 96 wells plate with single agent concentrations of HSPIs (12.5 μM) and Bortezomib (15.6 nM and 31.2 nM). Alternatively, one drug (one of the HSPIs or Bortezomib) was added to the cells for one hour; then the other drug (one of the HSPIs or Bortezomib) was added following this hour of treatment and the plate with K562 and U937 cells was incubated at 37 °C for 6 hours. To measure Bcl-2 levels, the treated cells were either probed with anti-Bcl-2 antibody, or were not probed to have a negative control for each treatment. A probed live untreated cells was also used to set a threshold: high signal intensity caused the shift to the right of such threshold. This happened on treated cells, for example. On the contrary, control with no antibody did not show a high intensity and there was no shift in respect of the threshold. The data for each triplicate of probed cells (treated cells and probe control) were collected and graphed using GraphPad Prism™ 6 version 6.05. Below there is an example of two different histograms which represent a control of cells with no FITC anti-Bcl-2 antibody and treated cells with FITC anti-Bcl-2 antibody. Purely as an example, the combination 12.5\31.2 of Pifithrin-μ and Bortezomib was chosen.

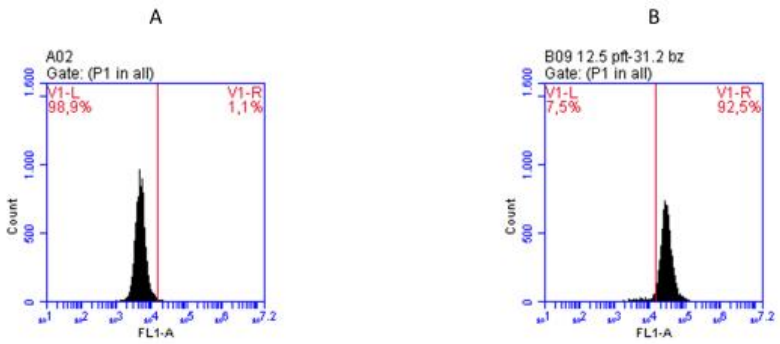


Fig 5.2.4.1. Example of histograms of Bcl-2 levels on flow cytometer: the first histogram represents live cells with no anti-Bcl-2 antibody; there is no signal and the cells do not pass the set threshold (A). On the contrary, the second histogram represents treated cells with FITC anti-Bcl-2 antibody; the population shifted to the right of the red line because of high signal intensity, indicating the Bcl-2 expression (B).

5.2.5. Statistical analysis

All the statistical analysis were performed according to what described on Chapter 2.3.8.

5.3. RESULTS

5.3.1. HSP72 expression following combined treatment of Pifithrin-μ and Bortezomib on K562 cells

Following one hour of treatment with Pifithrin-μ, Bortezomib was added for 6 hours to K562 cells to measure HSP72 inhibition. The inhibition of HSP72 was found in 95.93 % of the cells when Pifithrin-μ was added as single agent at 12.5 μM. When Bortezomib was added following an hour of Pifithrin-μ treatment, HSP72 was inhibited in 85.47 % of the cells (12.5\15.6 combination) and in 95.08 % of the cells when 31.2 nM was added. Similarly, when Bortezomib was added as single agent, HSP72 was inhibited in 95.01 % of the cells (15.6 n M) and in 89.08 % when the dose was 31.2 nM. All the concentrations were statistically compared to their respective control, resulting all significant different as ($P<0.0001$). Also, the drug concentrations were all compared to each other and no difference was found.

When Bortezomib was added first, the inhibition of HSP72 was not relevantly different from the experiment previously described. Indeed, when Bortezomib was added as single agent at 15.6 nM, HSP72 was inhibited in 96.80 % of K562 cells; Bortezomib at 31.2 nM inhibited 96.37 % of the cells. With respect of combined therapy, when 15.6 was added to 12.5 μM Pifithrin-μ following an hour, the cells where HSP72 was inhibited were 95.24 %. Also, the combination 12.5\31.2 showed a HSP72 inhibition in 97.19 % of the cells. When Pifithrin-μ was added as single agent the HSP72 inhibition was found in 94.82 % of the cells. All the concentrations were statistically compared to their respective control, resulting all significant different as ($P<0.0001$). Also, the drug concentrations were all compared to each other and no difference was found.

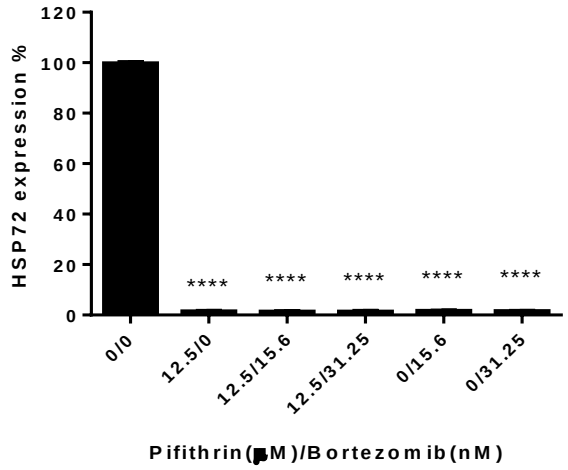


Fig. 5.3.1.1. HSP72 expression in K562 cells following 1h Pifithrin-μ and 6h Bortezomib combined treatment. K562 cells ($1x10^6$ cells\ml) were analysed on the flow cytometer following the combined treatment to investigate HSP72 expression. Data are presented as mean ± SD, n=3. **** (P<0.0001) using one way ANOVA Dunnett's multiple comparisons test. Data are all compared to live cells control untreated, which were expressing 100 % of HSP72. Following the treatment, the treated cells were not expressing HSP72, as suggested by the shifting downwards the FL1 channel on the flow cytometer. This indicated HSP72 inhibition.

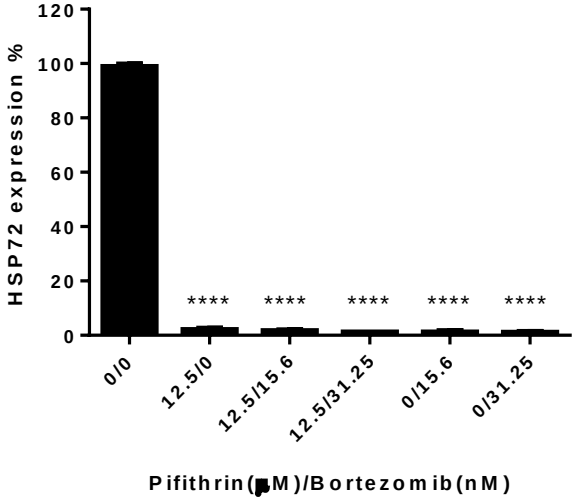


Fig. 5.3.1.2. HSP72 expression in K562 cells following 1h Bortezomib and 6h Pifithrin-μ combined treatment. K562 cells ($1x10^6$ cells\ml) were analysed on the flow cytometer following the combined treatment to investigate HSP72 expression. Data are presented as mean ± SD, n=3. **** (P<0.0001) using one way ANOVA Dunnett's multiple comparisons test. Data are all compared to live cells control untreated, which were expressing 100 % of HSP72. Following the treatment, the treated cells were not expressing HSP72, as suggested by the shifting downwards the FL1 channel on the flow cytometer. This indicated HSP72 inhibition.

5.3.2. HSP72 inhibition following combined treatment of PES-CL and Bortezomib on K562 cells

K562 cells were also treated with PES-CL and Bortezomib, in combination and as single agents. When PES-CL was added as single agent, 96.67 % of the cells showed HSP72 inhibition. Following an hour treatment with PES-CL, 15.6 nM Bortezomib was added to the PES-CL – treated cells; 89.06 % of the cells showed to have HSP72 inhibition. When 31.2 nM Bortezomib was added to PES-CL – treated cells, 96.81 % of them resulted to inhibit HSP70. As single agent, Bortezomib showed to inhibit 94.48 % of the cells (15.6 nM) and 96.36 % (31.2 nM). Similar to what performed with Pifithrin-μ\Bortezomib combination, the concentrations resulted statistically different as ($P<0.0001$) when they were compared to their respective controls. Also, when the drug combinations were compared to each other, they did not appear to be statistically significant (Fig. 5.3.2.1.)

When Bortezomib was administered first, the results did not differ from the previous experiment. Indeed, at 15.6 nM Bortezomib single agent 93.22 % of K562 cells appeared to inhibit HSP72. When Bortezomib was added as 31.2 nM, 93.95 % of the cells showed an HSP72 inhibition. Following an hour of Bortezomib treatment, PES-CL was added to the two concentrations. When PES-CL was added to 15.6 nM, HSP72 was inhibited in 94.65 % of K562 cells, whilst when PES-CL was added to 31.2 nM the HSP72 inhibition was found in 96.38 % of the cells. Also, when PES-CL was added as a single agent, HSP72 inhibition was detected on 94.42 % of K562 cells. Consistent to the previous experiments, the concentrations resulted statistically different as ($P<0.0001$) when they were compared to their respective controls. Also, when the drug combinations were compared to each other, they did not appear to be statistically significant (Fig. 5.3.2.2.)

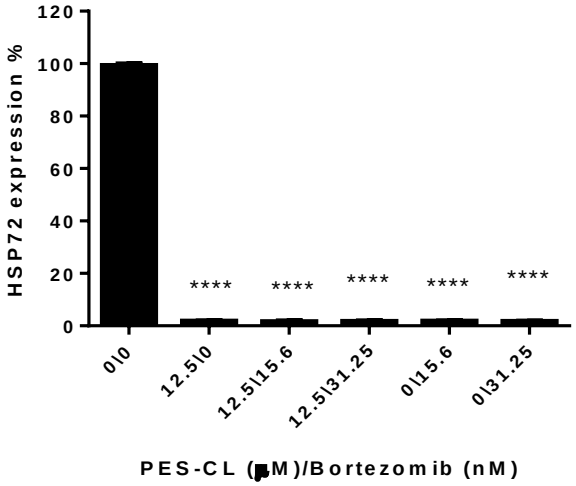


Fig. 5.3.2.1. HSP72 expression in K562 cells following 1h PES-CL and 6h Bortezomib combined treatment. K562 cells (1x10^6 cells\ml) were analysed on the flow cytometer following the combined treatment to investigate HSP72 expression. Data are presented as mean ± SD, n=3. **** (P<0.0001) using one way ANOVA Dunnett's multiple comparisons test. Data are all compared to live cells control untreated, which were expressing 100 % of HSP72. Following the treatment, the treated cells were not expressing HSP72, as suggested by the shifting downwards the FL1 channel on the flow cytometer. This indicated HSP72 inhibition.

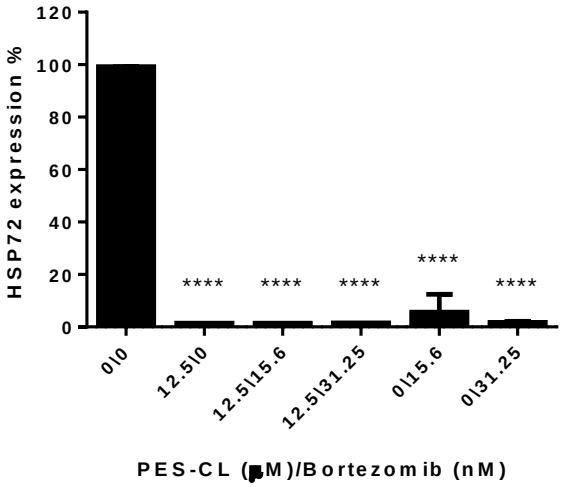


Fig. 5.3.2.2. HSP72 expression in K562 cells following 1h Bortezomib and 6h PES-CL combined treatment. K562 cells (1x10^6 cells\ml) were analysed on the flow cytometer following the combined treatment to investigate HSP72 expression. Data are presented as mean ± SD, n=3. **** (P<0.0001) using one way ANOVA Dunnett's multiple comparisons test. Data are all compared to live cells control untreated, which were expressing 100 % of HSP72. Following the treatment, the treated cells were not expressing HSP72, as suggested by the shifting downwards the FL1 channel on the flow cytometer. This indicated HSP72 inhibition.

5.3.3. Inhibition of HSP72 caused by combined therapy with Pifithrin-μ and Bortezomib on U937 cells

The inhibition of HSP72 caused by combined treatment of Pifithrin-μ and Bortezomib was measured on U937 cells. When Pifithrin-μ was added first, the results found did not differ much from what as been shown for K562. Indeed, when Pifithrin-μ was added as single agent, 93.32 % of the cells inhibited HSP70. When 15.6 nM Bortezomib was added to Pifithrin – treated cells, the HSP72 inhibition was detected on 89.51 % of the cells. U937 cells were also treated with 12.5\31.2 nM Bortezomib combination, resulting in a 91.93 % HSP72 inhibition. Bortezomib as a single agent induced HSP72 inhibition to 95 % of the cells at 15.6 nM concentration and to 89 % of U937 cells at 31.2 nM. Each control, specific to each drug combination, did not show any HSP72 inhibition. Consistent to the previous experiments, the concentrations resulted statistically different as ($P<0.0001$) when they were compared to their respective controls. Also, when the drug combinations were compared to each other, they did not appear to be statistically significant (Fig. 5.3.3.1.).

When Bortezomib was added first, the HSP72 levels of inhibition were high, consistent to what found in all the previous experiments. At 15.6 nM 93.03 % of the cells showed to have HSP72 inhibited, whilst at 31.2 nM the inhibition was found on 84.35 % of U937 cells. When 12.5 μM Pifithrin-μ was added following an hour of treatment with Bortezomib, the HSP72 inhibition levels were detected as 91.99 % (12.5\15.6) and 86.04 % (12.5\31.2). Consistently, each specific control did not show any HSP72 binding. Also, consistently with previous experiments, the concentrations resulted statistically different as ($P<0.0001$) when they were compared to their respective controls. Also, when the drug combinations were compared to each other, they did not appear to be statistically significant (Fig. 5.3.3.2.).

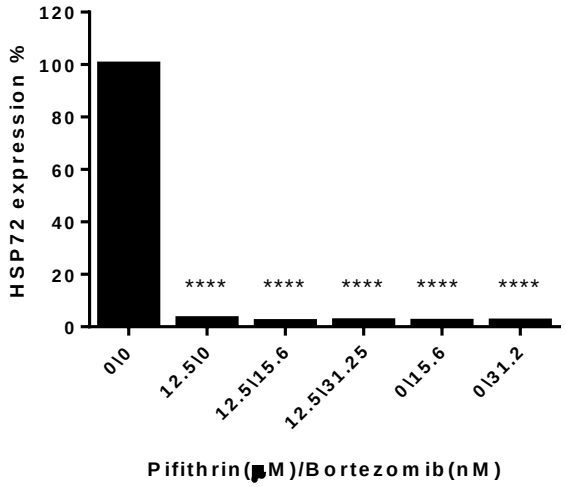


Fig. 5.3.3.1. HSP72 expression in U937 cells following 1h Pifithrin-μ and 6h Bortezomib combined treatment. U937 cells ($1x10^6$ cells\ml) were analysed on the flow cytometer following the combined treatment to investigate HSP72 expression. Data are presented as mean ± SD, n=3. **** (P<0.0001) using one way ANOVA Dunnett's multiple comparisons test. Data are all compared to live cells control untreated, which were expressing 100 % of HSP72. Following the treatment, the treated cells were not expressing HSP72, as suggested by the shifting downwards the FL1 channel on the flow cytometer. This indicated HSP72 inhibition.

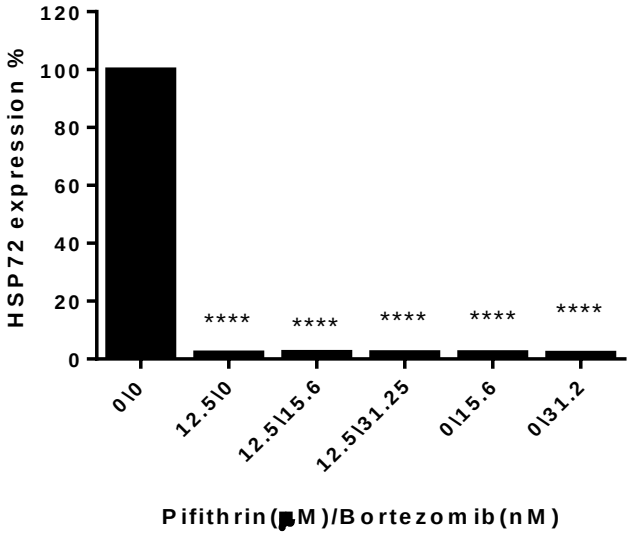


Fig. 5.3.3.2. HSP72 inhibition in U937 cells following 1h Bortezomib and 6h Pifithrin-μ combined treatment. U937 cells ($1x10^6$ cells\ml) were analysed on the flow cytometer following the combined treatment to investigate HSP72 expression. Data are presented as mean ± SD, n=3. **** (P<0.0001) using one way ANOVA Dunnett's multiple comparisons test. Data are all compared to live cells control untreated, which were expressing 100 % of HSP72. Following the treatment, the treated cells were not expressing HSP72, as suggested by the shifting downwards the FL1 channel on the flow cytometer. This indicated HSP72 inhibition.

5.3.4. Inhibition of HSP72 caused by combined therapy with PES-CL and Bortezomib on U937 cells

PES-CL single agent resulted to inhibit HSP72 to 96.13 % of U937 cells; when 15.6 nM Bortezomib was added following one hour of treatment with PES-CL, 89.05 % of U937 cells inhibited HSP72 activity. The 12.5\31.2 combination inhibited HSP72 to 90.56 % of cells, whilst 95.98 % of the cells inhibited HSP72 when Bortezomib was added as single agent at 31.2 nM. Also, 15.6 nM Bortezomib single agent inhibited the protein activity to 96.80 % of U937 cells. Generally, the combinations inhibited less cells than the single agent concentrations, although the inhibition was overall high. All the controls resulted not inhibiting HSP72 protein; only the control 12.5 µM resulted in 8.24 % of inhibition. Consistently to what found in the previous experiments, all the concentrations were compared to their respective control, resulting significantly different as ($P<0.0001$). When only the treated cells were compared between them, 12.5\0 resulted different as ($P<0.05$) when compared to 15.6 nM. The remaining concentrations were not significantly different (Fig. 5.3.4.1.).

When Bortezomib was added first as a single agent, the HSP72 inhibition was found in 91.86 % of U937 cells (15.6 nM) and in 87.06 % (31.2 nM). Following an hour treatment with Bortezomib, PES-CL was added; when in combination with 15.6 nM, 80.63 % of the cells inhibited PES-CL. The 12.5\31.2 combination resulted in an inhibition of HSP72 in 86.07 % U937 cells. When PES-CL was added as single agent, 89.77 % of cells inhibited the protein. Each control did not result inhibiting HSP72; also, the concentrations of treated cells did not result significantly different when compared to each other. Instead, the comparison between each control and its respective treatment resulted in a difference as ($P<0.0001$), as shown in Fig.5.3.4.2.

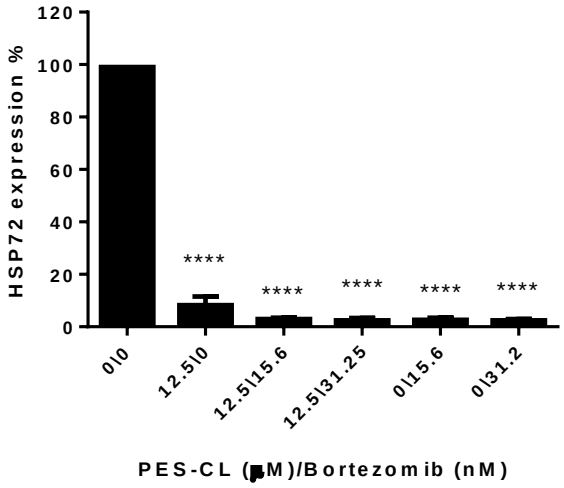


Fig. 5.3.4.1. HSP72 expression in U937 cells following 1h PES-CL and 6h Bortezomib combined treatment. U937 cells (1x10^6 cells\ml) were analysed on the flow cytometer following the combined treatment to investigate HSP72 expression. Data are presented as mean ± SD, n=3. **** (P<0.0001) using one way ANOVA Dunnett's multiple comparisons test. Data are all compared to live cells control untreated, which were expressing 100 % of HSP72. Following the treatment, the treated cells were not expressing HSP72, as suggested by the shifting downwards the FL1 channel on the flow cytometer. This indicated HSP72 inhibition.

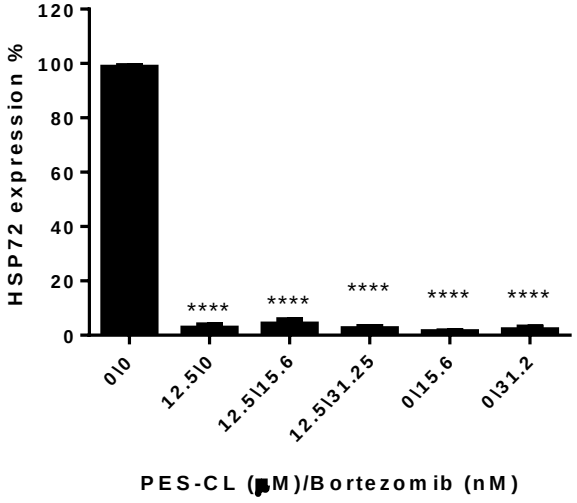


Fig. 5.3.4.2. HSP72 expression in U937 cells following 1h Bortezomib and 6h PES-CL combined treatment. U937 cells (1x10^6 cells\ml) were analysed on the flow cytometer following the combined treatment to investigate HSP72 expression. Data are presented as mean ± SD, n=3. **** (P<0.0001) using one way ANOVA Dunnett's multiple comparisons test. Data are all compared to live cells control untreated, which were expressing 100 % of HSP72. Following the treatment, the treated cells were not expressing HSP72, as suggested by the shifting downwards the FL1 channel on the flow cytometer. This indicated HSP72 inhibition.

5.3.5. Bcl-2 expression on U937 cells following combined therapy with Pifithrin-μ and Bortezomib

Following an hour of treatment with Pifithrin-μ, 15.6 nM and 31.2 nM Bortezomib was added to the cells for 6 hours and Bcl-2 levels were measured on the flow cytometer. Bcl-2 was found in 68.16 % of U937 cells when Pifithrin was added alone and in 57.8 % of cells when 15.6 nM Bortezomib was added as single agent, resulted significant as ($P<0.05$). At 31.2 nM Bcl-2 was found in 64.22 % of the cells, whilst when it was added to Pifithrin-μ treated cells the percentage found was of 60.86 % ($P<0.05$). The lowest percentage of Bcl-2 levels was found when 15.6 nM Bortezomib was added following an hour of Pifithrin-μ treatment ($P<0.05$); 53.68 % of U937 cells expressed Bcl-2 protein following this combination. The treated cells were also all compared to each other and no significant difference was found, as shown on Fig. 5.3.5.1.

Similar analysis was performed on the flow cytometer when Bortezomib was added for an hour and Pifithrin-μ was subsequently added for 6 hours. As single agent 15.6 nM Bortezomib induced Bcl-2 expression in 69.67 % ($P<0.05$) of the cells, whilst the protein was found in 48.90 % of U937 cells at 31.2 nM Bortezomib single agent ($P<0.001$). Interestingly, similar values were found when Pifithrin-μ was added to 15.6 nM and 31.2 nM; Bcl-2 was found in 64.56 % of the cells at 12.5\15.6 ($P<0.05$) and in 64.89 % ($P<0.05$) of cells at 12.5\31.2. Lastly, 12.5 μM Pifithrin-μ single agent induced Bcl-2 expression in 59.93 % ($P<0.01$) of U937 cells. Consistently to what mentioned in the previous paragraph, the treated cells were also compared to each other and no statistical significant difference was found (Fig. 5.3.5.2.).

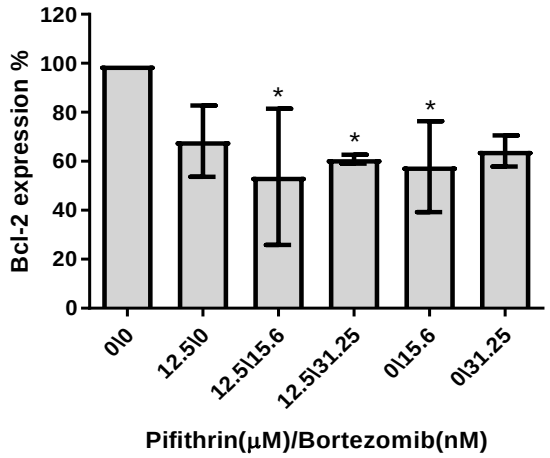


Fig. 5.3.5.1. Bcl-2 expression in U937 cells following 1h Pifithrin-μ and 6 h Bortezomib combined treatment. U937 cells ($1x10^6$ cells\ml) were treated with the drug combinations and Bcl-2 levels were measured as % of cells expressing the protein following the treatment. Data are presented as mean ± SD, n=3. *(P<0.05), using one-way ANOVA Dunnett's multiple comparisons test. Data are all compared to live cells control untreated.

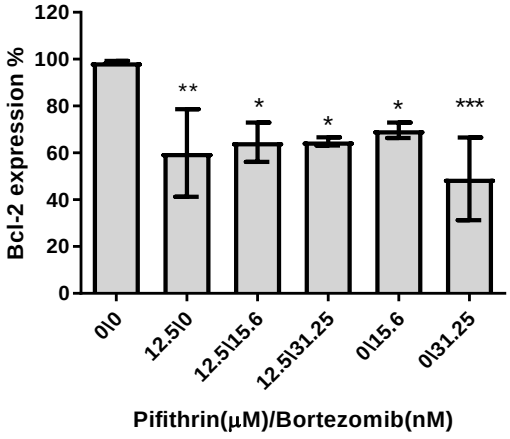


Fig. 5.3.5.2. Bcl-2 expression in U937 cells following 1h Bortezomib and 6h Pifithrin-μ combined treatment. U937 cells ($1x10^6$ cells\ml) were treated with the drug combinations and Bcl-2 levels were measured as % of cells expressing the protein following the treatment. Data are presented as mean ± SD, n=3. *(P<0.05), ** (P<0.01), *** (P<0.001) using one-way ANOVA Dunnett's multiple comparisons test. Data are all compared to live cells control untreated.

5.3.6. Bcl-2 expression in U937 cells following combined therapy with PES-CL and Bortezomib

Levels of Bcl-2 protein were measured on U937 cells on the flow cytometer following a subsequent combined therapy with PES-CL and Bortezomib. PES-CL was added for an hour as a single agent and Bcl-2 was found on 91.24 % of the cells; when 15.6 n M Bortezomib was added following an hour of PES-CL treatment, Bcl-2 was found on 93.27 % of U937 cells. On the other combination 12.5\31.2, the protein was expressed in 93.30 % of the cells. Less protein was found when Bortezomib was added as single agent, although still relevant; Bcl-2 was found in 82.17 % of cells at 15.6 nM ($P<0.01$) and in 85.33 % of cells ($P<0.05$) at 31.2 nM. (Fig. 5.3.6.1.)

Similar approach was taken to measure Bcl-2 levels when Bortezomib was added first followed by 6 hours treatment with PES-CL. 15.6 n M Bortezomib as single agent resulted to induce Bcl-2 expression in 40.97 % of U937 cells ($P<0.0001$), whilst the combination with 12.5 µM PES-CL resulted in Bcl-2 expression in 60.76 % ($P<0.01$). An equal result was found on the combined concentration 12.5\31.2, where Bcl-2 was found in 60.96 % of U937 cells ($P<0.01$). When 31.2 n M Bortezomib was added as a single agent, Bcl-2 was found in 67.07 % ($P<0.05$) of the cells. Lastly, 91.06 % of U937 cells expressed Bcl-2 when PES-CL was added as single agent. The treated cells with stain were also all compared between them, again without showing any statistical significant difference (Fig. 5.3.6.2.)

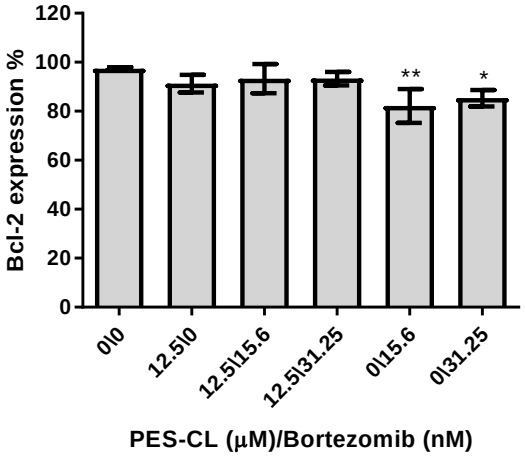


Fig. 5.3.6.1. Bcl-2 expression in U937 cells following 1h PES-CL and 6 h Bortezomib combined treatment. U937 cells (1x10^6 cells\ml) were treated with the drug combinations and Bcl-2 levels were measured as % of cells expressing the protein following the treatment. Data are presented as mean ± SD, n=3. *(P<0.05), ** (P<0.01), using one-way ANOVA Dunnett's multiple comparisons test. Data are all compared to live cells control untreated.

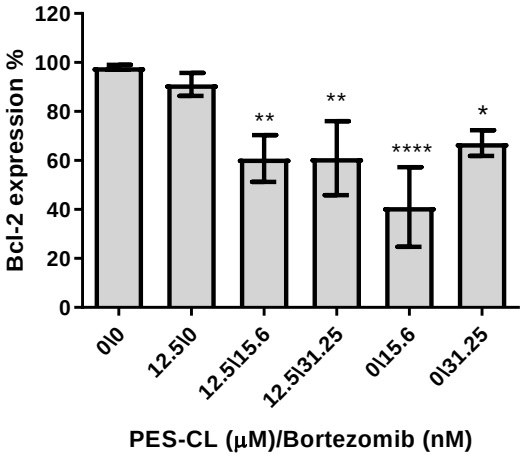


Fig. 5.3.6.2. Bcl-2 expression in U937 cells following 1h Bortezomib and 6h PES-CL combined treatment. U937 cells (1x10^6 cells\ml) were treated with the drug combinations and Bcl-2 levels were measured as % of cells expressing the protein following the treatment. Data are presented as mean ± SD, n=3. *(P<0.05), (P<0.01), (P<0.0001), using one-way ANOVA Dunnett's multiple comparisons test. Data are all compared to live cells control untreated.

5.4. DISCUSSION

5.4.1. HSP72 inhibition on K562 and U937 cells following combined treatment with HSPIs and Bortezomib

The combination between Pifithrin-μ and Bortezomib resulted in an inhibition of HSP72 on both combinations and also when the drugs were added as single agents. Importantly, for example on K562 cells, the results were similar independently from single agent administration or combination, when Bortezomib was added first (Fig.5.3.1.2.). There was no difference between the different treatments, suggesting two main conclusions: firstly, Bortezomib and Pifithrin-μ seems to strongly interact in a specific fashion with HSP72, therefore potentially inhibiting its activity. Secondly, Pifithrin does not seem to enhance Bortezomib activity. A partial confirm of the interfering activity of Pifithrin-μ could be found in the study by Ishaq M., *et al*., (2016). In human bladder cancer cells the combination between Pifithrin-μ and gambogic acid (GA) switched the cell death from caspase dependant apoptosis to caspase independent apoptosis. Interestingly, GA did not specifically bind to HSP72 as Bortezomib seems to do; therefore, the targets of these two drugs could be different and their action could potentially be synergic. Also, GA normally induces caspase-dependant apoptosis on leukemic cell lines, normally detected by Annexin V channels on the flow cytometer, as also stated by Gausdal G., *et al*., (2004). Also, similarly to this study, there was no synergy between the two drugs; this further confirms that Pifithrin-μ could play a role an important role in apoptosis, but not when in combination with other drugs. To note, Ishaq M., *et al*., (2016) have demonstrated the possible antagonistic effect of Pifithrin-μ on bladder cancer cell lines on a 24 h time course and using higher and increasing concentrations of Pifithrin-μ, 20 μM and 40 μM. It is not clearly stated the reason of the choice of such concentrations and the time of treatment, as it is attempted to do in this thesis. However, this research indicates also that the inhibition of HSP72 could affect the caspase – dependant signalling cascade, leading to an independent apoptosis type of cell death. Bladder cancer cells were treated with GA and Pifithrin-μ for 24 hours (40 μM again) following a treatment with z-VAD-fmk (a pancaspase inhibitor), showing no effect on cell death whether on combined therapy or single agents; this indicates that Pifithrin-μ and the HSP70 inhibition could play an important role in the independent cell death in bladder cancer cells. However, there was no significant difference between all the drug concentrations; therefore, the HSP72 inhibition was caused in high percentage and it is difficult to fully understand how antagonistic these drugs could be in respect of HSP72 inhibition.

U937 cells were more responsive than K562 cells to the treatment in terms of apoptosis percentages and effects on cell viability (Fig.5.3.3.2.). The results of this study tend to agree with what has been demonstrated by (Leu J.I., *et al*., 2011). Indeed, the drugs Pifithrin-μ and Bortezomib were tested together in combination and separately on two different cell lines (H1299 and A875 cells). The results have shown an interfered proteasome activity following the combination of Pifithrin-μ and Bortezomib; instead, proteasome activity was normally disrupted when Pifithrin-μ and Bortezomib were added to the cells separately. The 20s proteasome cleavage suggested that the combination of these two drugs do not affect proteasome activity, whether Pifithrin-μ is able to disrupt proteasome activity equally to a proteasome inhibitor like Bortezomib and EGCG, like suggested by the fluorogenic assay to measure 20s intensity. The treatment was 24 hours long and not 6 hours like in

this thesis and the doses were different (20 μM for Pifithrin-μ and 100 n M for Bortezomib). To note that this thesis measured the HSP72 inhibition on the flow cytometer only, whether the research mentioned above also measured proteasome activity using different techniques such as the fluorogenic assay and western blot. Also, the results of this study could perhaps be important to understand that HSP72 inhibition occurred in such a shorter time of treatment (6 hours and not 24 hours) with lower concentrations. Naturally, the cell lines that have been used by these researchers are different from K562 cells and U937 cells; however, the results seem to be in agreement with what found by (Leu Ji., *et al*., 2011).

PES-CL resulted to inhibit HSP72 expression almost completely on both cell lines, with no statistical difference between single and combined treatment (Fig.5.3.4.1. and Fig.5.3.4.2.). Interestingly, this seems to find support in the work by Yerlikaya A., Okur E., Eker S. and Erin N., (2010). Bortezomib has been tested with other HSP70 inhibitors such as quercetin, KNK-437 and schisandrin-B on melanoma cancer cell lines. Differently from this study, the combined therapy was performed over 48 hours and the drugs were not added at different times. Following western blot experiments, it was determined that 20 μM quercetin and 10 nM Bortezomib did not affect proteasome activity whilst these two concentrations alone; in fact, the HSP70 inhibitor seemed to inhibit the apoptosis effect of bortezomib, confirming the competitive action of HSP70 inhibitor on Bortezomib. Interestingly, quercetin seemed to enhance the apoptosis activity of another proteasome inhibitor called MG-132, which was added with quercetin and with the combination quercetin\bortezomib, on a very low dose (0.5 μM). Although the cell lines are obviously different and they represent different type of cancer, and there was no investigation on the flow cytometer to understand the percentage of cells inhibiting HSP70, it could be indicative to confirm that HSP70 inhibitors and Bortezomib have an antagonistic effect and that HSP70 inhibition does not necessarily enhance proteasome activity. KNK-437 and Schisandrin-B were also tested in combination with Bortezomib, confirming that HSP70 inhibition did not improve Bortezomib activity. However, only cell viability was measured in this research, limiting the comparison with this study (Yerlikaya A., Okur E., Eker S. and Erin N., 2010).

5.4.2. Bcl-2 expression on U937 cells following combined treatment with HSPIs and Bortezomib

Interestingly, Bcl-2 expression decreased when Pifithrin-μ and Bortezomib were combined; it appeared that the drug subsequently added could not improve the results of the drug firstly administered as single agent. For example, when Bortezomib was added first the combination with Pifithrin-μ induced Bcl-2 expression to 64 % of the cells on both combined concentrations. The result of single agent administration of Bortezomib 15.6 n M, for example, was that 69 % of U937 cells expressed the protein (Fig.5.3.5.2.) Although slightly higher, this result was compared to the others treatment, showing no significant difference. The failed inhibition of Bcl-2 by Pifithrin-μ and Bortezomib could find partial confirmation in what proposed by (Zhuang Y., Berens-Norman HM., Leser JS., Clarke P. and Tyler KL., 2016) showed that in order to prevent apoptosis and tissue damage during viral encephalitis, Pifithrin-μ can decrease the formation of p53/Bak complexes, avoiding apoptosis following reovirus infection of ex vivo brain slice cultures. With this in mind, it appears clearer why Bcl-2 is strongly expressed following Pifithrin-μ treatment.

Another interesting study by (Drakos E. *et al*, 2010) showed that 4.8 μ of Pifithrin-μ rescued a relevant percentage of nutlin-3a-treated cells (large B-cell lymphoma) from apoptotic cell death, according to their Annexin V analysis. Howewer, Pifithrin-μ did not inhibit the release of cytochrome c and therefore the accomplishment of apoptosis when Bcl-2 protein was silenced by Bcl-2 inhibitors. The analysis proposed in this study is based solely on flow cytometry experiments, which measured Bcl-2 expression on U937 cells. Although the difference in the target, it suggested that Pifithrin-μ could evidently induce apoptosis when Bcl-2 was not expressed and could even promote survival in presence of nutrin. This is in agreement with what suggested by this study, where the Bcl-2 levels are high following combined treatment and single agent treatments, but there is still apoptosis following 24 hours or 6 hours of treatment. However, the small dose of Pifithrin-μ resulted surprising; perhaps it could be suggested that AML cells may require higher doses of treatment. The time of treatment was also not fully and clearly specified, limiting the comparison.

PES-CL – Bortezomib experiments showed that Bcl-2 is not inhibited by single agent or combined therapy when PES-CL was administered first. Bcl-2 resulted highly expressed when PES-CL was administered first, showing no difference between single agent or combination treatment (Fig.5.3.6.1.). Interestingly, when Bortezomib was administered first, there was a reduction in Bcl-2 expression; this was particularly evident when Bortezomib was added as single agent at 15.6 nM (Fig.5.3.6.2.) Thus, PES-CL seems to interfere with Bortezomib mechanism of action; perhaps one hour of PES-CL treatment is potent enough to completely inhibit HSP72 as shown previously, which cannot longer assist the proteasome in disrupting Bcl-2 mechanism. On the contrary, one hour of Bortezomib treatment alone is enough for Bortezomib to bind to proteasome, reducing Bcl-2 levels, although not completely. This indicated that Bortezomib may require a longer time of treatment than one hour or six hours when combined to completely inhibit Bcl-2 mechanism. Therefore, Bcl-2 expression results may suggest that the cells could affect mitochondrial pathway, inhibiting caspase activation. Bcl-2 high levels described in this chapter may suggest the involvement of an extrinsic pathway of apoptosis, which may involve the inhibition of NFκB pathway preceded by a caspase 8\10 activation as suggested by Krakstad, C., and Chekenya, M., (2010). The reduced expression of Bcl-2 when Bortezomib is administered first with both HSPIs may support this model; indeed, Bortezomib appeared to inhibit NFκB pathway on mice (Hsu, S. M., *et al*., 2015). In partial support of the results of this chapter, Lauricella M. *et al*, (2006) demonstrated that Bortezomib is able to reduce Bcl-2 activity on hepatoma cells; however, the cells death is associated with high level of pro-apoptotic proteins and activation of mitochondrial pathway. The Italian group of researchers also proposed a mechanism involving FasL/caspase-8, following changes on Bcl-2 levels, which may agree what is proposed in this chapter.

There is a limited literature on PES-CL mechanism of action on leukemic cell lines; however, the combination between PES-CL and Bortezomib and the high level of Bcl-2 as a consequence may be in partial agreement with what found by Chauhan *et al*. (2008). Following treatment with 10 nM of Bortezomib and 10 nM of NPI-0052 for 24 hours on multiple myeloma cells, the role and involvement of caspase 8 and caspase 9 was evaluated. Indeed, pan-caspase, caspase-8 or caspase-9 inhibitors were added to the cells with the proteasome inhibitors and cell viability was measured. The analysis revealed that Bortezomib apoptosis was induced by mediation of caspase 8 and caspase 9; indeed, when Bortezomib or the other proteasome inhibitor were in presence of the caspases inhibitor the cell viability levels were incredibly higher if compared to the levels of cell viability when there was no caspase inhibitor. Although, there was no addiction of caspase inhibitors, high

expression of Bcl-2 following PES-CL and Bortezomib treatment are supported by these findings, especially considering the experiment where PES-CL was administered first. However, when Bortezomib treatment was analysed on western blot with Bax antibody, the mitochondrial extracts revealed an important accumulation; this suggests that Bcl-2 has been somehow inhibited by Bortezomib, thus not find any association with their previous results. However, this is in agreement with what found in this chapter when Bortezomib was administered first. Indeed, the reduction of Bcl-2 levels, although partial, may be associated with an increase of pro-apoptotic protein. This needs further confirmation in a potential future study. It also surprised that 10 nM could affect significantly the cells following 24 hours, although the data were not clearly stated (Chauhan *et al*., 2008).

The inhibition of HSP72 by Bortezomib in particular did not completely affect Bcl-2 levels, despite the promising results shown on the HSP72 inhibition experiment. This finds partial confirm in what suggested by Pei X.Y., *et al*., (2003) who tested multiple myeloma cells with and without Bcl-2 inhibitor HA14-1. The Annexin V\PI analysis clearly indicated an improvement in apoptosis levels when the Bcl-2 inhibitor was added; importantly, a western blot analysis revealed that alternated subsequent administrations of HA14-1 and Bortezomib induced cytosolic release of proteins like cytochrome c and DIABLO which are a proof of mitochondrial permeabilization and, therefore, mitochondrial pathway. This is in partial agreement with what showed with Pifithrin-μ and Bortezomib treatment; there was a reduction of Bcl-2 expression, independently from which drug was administered first. Indeed, there was no significant difference between the whole range of concentration on the two experiments, indicating that one hour of treatment with a drug may be enough to antagonize the other drug, similarly to what proposed with PES-CL. Although relevant differences between the two studies, such as different techniques, cell lines and different treatments, there is the indication that when Bcl-2 is not inhibited, like in the results presented in this chapter, there is no mitochondrial damage. When Bcl-2 is inhibited, there is the release of pro-caspase proteins, sign of apoptosis activated by mitochondrial damage (Pei X.Y., *et al*., 2003).

Taken together, these results show that PES-CL and Pifithrin-μ are able to bind to HSP72, strongly inhibiting its chaperone activity and therefore stopping Bcl-2 disruption in the proteasome. Particularly, PES-CL administered first appear to completely stop Bcl-2 cleavage, confirming that it is a more potent drug than Pifithrin-μ. On the contrary, when Bortezomib is administered first, one hour of treatment is not enough to affect Bcl-2 expression, suggesting that a longer time of treatment may perhaps completely lead to Bcl-2 cleavage. Therefore, it is proposed that the cells die by an apoptosis mechanism which is independent by caspase signalling cascade. However, it is not still clear if these drugs always cause apoptosis independently from the mitochondrial involvement, as it is also shown above by part of the literature. Perhaps, 6 hours is not a sufficient time to trigger the release of cytochrome c and the consequent caspases 3-7 signals cascade; indeed, this could potentially explain the Bcl-2 expression. To further evaluate this, future experiments could point to perform the same experiments with a longer treatment and, also, possibly investigate the expression of pro-apoptosis protein such as Bax and Bak.

CHAPTER 6: DISCUSSION AND CONCLUSION

6.1. Discussion

Cancer can be considered as one of the most important disease of the past century, not only for the importance that it undoubtedly plays in our society, but also for the constant discoveries that are made in the attempt to find cure for all the types of cancer. Chemotherapy is the most common type of treatment and research has made several positive steps toward curing the disease. Further, the aim of oncologists and researchers is to specifically kill cancer cells while minimising damage to normal cells. What was probably a utopic dream a few decades ago, where chemotherapy was aiming to kill all the tumour cells, it is now possible (Flaherty, K. T., 2006). On CML and AML the need of a more selective treatment has led to the development of the first new biological drugs; the first one ever created was Imatinib, which is still currently in use for the treatment of these types of leukaemia (Druker B.J. *et al*, 2006 and Hochhaus, A. *et al*, 2017 and Barratt, D. T., & Somogyi, A. A., 2017). A TT drug is extremely selective for their target; particularly, it would specifically aim to restore apoptosis signalling cascades in cancer cells. This is fundamental for the choice of treatment and mostly for the health of a patient, which would avoid side effects given by chemotherapy or it would have reduced side effects (Stone R.M. *et al*, 2017 and Langer, S. W., 2014).

The overall aim of this thesis was to investigate the effects of a combination therapy on cell viability and apoptosis on leukemic cell lines. On leukaemia, one of the factors associated with poor prognosis is the overexpression of HSP protein, particularly HSP70 and HSP90 (Murphy M.E., 2013). Also, overexpression of HSP70 was found on BCR-ABL cells, leading to resistance to Imatinib treatment (Pocaly M. *et al*, 2007). HSP70 is also overexpressed on its stress-inducible form HSP72 on melanoma and on prostate cancer (Lazaris A.C., *et al*., 1995 and Abe M., *et al*., 2004). Also, the overexpression of HSP72 on mice model has been documented (Seo J.S. *et al*, 1996 and Volloch V.Z. and Sherman M.Y., 1999). The HSPs main role is a chaperone role, which means that they assist the correct folding or elimination of proteins by recognizing the polypeptides that need to be degraded. These proteins, through a process known as ubiquitination (from the protein ubiquitin) and through the assistance of HSPs, are destined to the proteasome where they are degraded. In cancer, the proteasome is not able to maintain the normal and appropriate levels of intracellular protein, leading to higher levels or pro-survival proteins than pro-apoptotic proteins, ultimately causing cell survival and tumour growth (Esser, C., *et al*., 2004 and Voorhees, P. M., *et al*., 2003). Disfunction in normal proteasome activity has been found on leukaemia, on AML for example (Csizmar, C. M., *et al*., 2016 and Niewerth, D., *et al*., 2013). Abnormal proteasome activity has also been found on pancreatic cancer and on multiple myeloma (Bold, R. J., *et al*., 2001 and Obeng, E. A., *et al*., 2006). The proteasome became therefore a target for cancer therapies, considering its crucial role in the cell survival and resistance to treatment. The proteasome inhibitors have been developed to re-establish the normal proteasome activity by inducing apoptosis which leads to the disruption of the pro-survival proteins (Adams J. *et al*, 1999).

With this in mind, it seemed interesting to further understand if these two targets can be successfully candidate for targeted therapy in a combination. More specifically, could designed HSPIs and designed PIs affect cell viability and induce apoptosis to leukemic cells? Also, could these drugs kill leukemic cells with small doses, attempting to be as selective and less toxic as possible? This thesis attempts to answer to these questions, also suggesting a proposed mechanism of apoptosis.

Initially, two leukemic cell lines representing two types of leukaemia (Chronic Myeloid Leukaemia and Acute Myeloid Leukaemia) were chosen. The use of human cell lines is crucial for *in vitro* experiments, because it allows to mimic what could potentially happen *in vivo*, whether on animals or on human patients. One cell line originated by a patient with Chronic Myeloid Leukaemia (CML) is K562; it was first studied in 1979 by Andersson, L. C., *et al*., (1979), who first described the characteristics of the cell line and it has been widely used in the following decades (Law J.C., *et al*., 2016). An important characteristic which led to the choice of this cell line is the percentage of blasts (> 30 %), which indicate a blast crisis. It therefore seemed interesting to evaluate the effects of treatment on such a critical state of CML, hoping to use the results for an eventual new treatment option. U937 cells are cells isolated from a patient with histiocytic lymphoma by Sundstrom C., (1976) and extensively used to study monocyte differentiation in diseases with myeloid lineages, such as Acute Myeloid Leukaemia, which is the reason why this cell line has been chosen. Similar to K562, U937 cells have been studied and analysed throughout the decades to understand the production of cytokines, for example, Biswas P. *et al*, (1998) or to understand the effectiveness of drug combinations, for example (Nowak M. *et al*, 2017).

Following the choice of the cell lines, the next step was to determine the type of treatment. As mentioned above, the investigation focused on HSP70 and the proteasome. Therefore, HSP70 and proteasome inhibitors needed to be considered; the drugs used and chosen were two HSPIs (Pifithrin-μ and PES-CL) and a proteasome inhibitor Bortezomib. Pifithrin-μ was initially designed as a p53 inhibitor, but it showed to have an antileukemic activity as HSP70 inhibitor (Kaiser M. *et al*., 2011 and Vaseva, A. V., & Moll, U. M., 2009 and Green, D. R., & Kroemer, G., 2009). PES-CL is a derivative of Pifithrin-μ and it has shown to be more potent than the parent molecule on leukaemia (Budina Kolomets A. *et al*, 2014 and Balaburski G.M. *et al*, 2013). Bortezomib is one of the commonest agent used in multiple myeloma treatment (Richardson P.G. *et al*, 2005 and Moreau P. *et al*, 2011); recently, it also demonstrated to be effective in leukaemia treatment (Satou, Y., *et al*., 2004 and Horton, T. M., *et al*., 2006). However, before attempting to treat cells with a combination of drugs, the first aim of the thesis was to analyse these drugs firstly as a single agent. HSPIs and Bortezomib were administered to K562 cells and to U937 cells for 24 hours and cell viability was measured via MTS assay. The range of drug concentrations initially started with high dilutions in order to determine the lowest concentrations that could ideally be considered for single agent treatment on a potential patient. This resulted in the individuation of three lowest dilutions for Pifithrin-μ and PES-CL, which were 50 μM, 25 μM and 12.5 μM on both cell lines (Fig. 3.3.1.1. and Fig. 3.3.1.2.). These results partially agreed with a study by Kaiser *et al*, (2011) which demonstrated that Pifithrin-μ affected cell viability on K562 cells on lower doses but on a longer time of administration. These results indicate that 24 hours may be enough to severely affect cell viability on leukemic cell lines, although there has not been found a further confirmation on literature with regards of the drug concentration.

Also, PES-CL generally resulted more effective than Pifithrin-μ on both cell lines, with particular emphasis on U937 cells (Fig. 3.3.4.2.) where the three concentrations mentioned above killed all the leukemic cells following 24 hours of treatment. PES-CL has not been extensively tested on leukemic cell lines, although it has been administered to myeloma cell lines (Budina-Kolomets, A., *et al*., 2014). The lack of literature on leukaemia is relevant, however the results of this study confirmed with the literature just mentioned on the fact that PES-CL seemed to be highly effective, more than the progenitor Pifithrin-μ. It is interesting to note that U937 seemed to respond better to treatment than K562 cells, especially following PES-CL treatment. The fact that K562 cells represent a CML patient in blast crisis, therefore with a blast percentage of more than 20 % in the bone marrow, may perhaps be the explanation of the more resistance to treatment if compared to U937 cells; the literature currently gives many examples of patients in blast crisis who are treated with various therapies to fight the drug resistance to classic treatments such as Imatinib (Saußele S. and Silver R.T., 2015). Therefore, it could be speculated that, given the higher percentage of blasts in the bone marrow on K562 cells and their tendency to be more resistant to treatment if compared to U937 cells (Kaiser *et al*., 2001), a more aggressive treatment with higher doses or longer time course could give better results in terms of apoptosis induction.

Following the same approach and principles of the previous paragraph, Bortezomib resulted effective on both cell lines; the initial range of concentrations indicated that 15.6 nM and 31.2 nM were the lowest concentration that may be used as single agent treatment for 24 hours (Fig. 3.3.5.1. and Fig. 3.3.5.2.). This drug is frequently administered to multiple myeloma patients (Kouroukis, T. C., *et al*., 2014), however it has been recently considered as potential treatment option for leukaemia. A study by Klikova K. *et al*. (2015) contradicts what has been found on this study; in fact, Bortezomib was tested on K562 cells for 24 hours with a range from 100 nM to 10 nM. Bortezomib did not affect cell viability on any of the concentrations of the range. This is opposite to the findings of this thesis, which demonstrated that even the minimum dose (15.6 nM) could reduce metabolic activity on K562 cells. However, lack of literature on leukemic cell lines may be filled by the results of this research and could potentially be a substrate for other researches. However, the results of these experiments are to consider as an initial step to the understanding of the effects of such drugs on the metabolism of CML and AML cell lines, which are described below.

The following questions that emerged were: do these drugs as single agent affect cell viability on a shorter time of treatment? How does the shorter time of treatment affect apoptosis levels? The HSPIs were added to both K562 cells and U937 cells every hour up to six hours at the lowest concentrations which were chosen following the results of the previous experiments with longer time of treatment. Interestingly, six hours resulted to have effects on cell viability on both cell lines, affecting more than 50 % of the cells on both cell lines. Also, following one hour of treatment, cell viability seemed to be affected, although at a minimal percentage (Fig. 3.3.2.1. and Fig. 3.3.3.1.). These results are potentially the first to indicate an initial response to low doses of HSPIs following a short period of treatment on leukemic cell lines. This was particularly important considering the potential implications that a short time of treatment on low dose such as 12.5 μM could have. A similar study by Monma *et al*, (2013) indicated that 5 hours of treatment with Pifithrin-μ can be effective on cell viability; although the experiments were performed on pancreatic cancer cell lines, these results together with what found on this study clearly indicate that Pifithrin-μ is a potential and promising therapy for different type of cancer. Considering the approach indicated at the beginning of the chapter, finding a potential treatment in common for different type of tumours is

key; this suggests that HSPs and HSP70 in particular could be inhibited in a short time with a similar pattern on different type of cells, with consequential different characteristics.

The effects on cell viability needed to be confirmed by analysing the apoptosis levels on both cell lines; indeed, it was interesting to understand if the cells were dying by apoptosis or necrosis, which was immediately excluded after the analysis. Very interestingly, the apoptosis levels following 6, 3 and 1 hour treatment with Pifithrin-μ confirmed that more than 30 % of the cells start to die by apoptosis throughout the time course (Fig. 3.3.2.2. and Fig. 3.3.3.2.); following these analysis, it appeared that there is no significant difference between the three doses and that there is no significant difference between the shortest time of treatment, which are three hours and an hour (Table 3.3.2.1. and Table 3.3.3.1.). Although a different drug, the effects on apoptosis of Pifithrin-μ can be compared to what induced by Pifithrin-α; Ng, L. T., & Wu, S. J. (2011) indicated that it had an important effect on apoptosis on human hepatoma Hep G2 cells, confirming the effectiveness of Pifithrin-μ in apoptosis, particularly on the inhibition of Bcl-xl. Also, Pifithrin-α showed to induce apoptosis on mice with hepatectomy, not inhibiting liver regeneration; this confirms the tendency of Pifithrin-μ to induce apoptosis, although it is not clear how long they treated the mice (Eipel, C., *et al*., 2005). These results are perhaps the first to indicate that at 12.5 μM and following one hour of treatment with Pifithrin-μ, CML and AML cell lines start to die by apoptosis. There is no similarity in any study, considering this dose and this time of treatment. Perhaps following 3 hours or 1 hour of treatment, HSP70 intracellular levels are almost completely inhibited, which may explain how a short time of treatment could start to induce apoptosis. However, the apoptosis levels may indicate only the state of a cell; despite being a remarkable result, it needed further clarification, which is described in the following paragraphs.

Based on a clinical trial on prostate cancer patients (Papandreou C.N. *et al*, 2004) and on clinical trial on lymphomas (Hamilton A.L. *et al*, 2005) who tested intravenous Bortezomib for one hour obtaining encouraging results on proteasome inhibition and also following what suggested by Chen, D., *et al*., (2011) and by the manufacturer (Merck Millipore), Bortezomib was administered to K562 and U937 cells for one hour. The Annexin V\PI indicated that Bortezomib killed by apoptosis more than half of the cells on K562 cells (Fig. 3.3.7.1.) and more than 60 % on U937 cells (Fig.3.3.7.3.). This result represent a novel therapy options with respect of CML and AML; Bortezomib has been administered for one hour intravenous on multiple myeloma patients (Jung, J. Y., *et al*., 2014), but there is a lack of literature on CML and AML and this work could potentially be beneficial as it potentially indicates a new treatment option. The short time of treatment could potentially be less toxic for patients who would have a reduced time of treatment and with the possibility of an immediate action on killing leukemic cells. However, K562 cells is more resistant to Bortezomib treatment than U937, which is consistent to what was previous described. In this sense, the sensibility could possibly be explained by the severity of the stage, as previously suggested and consistently found here, where perhaps a more aggressive approach needs to be considered.

The second aim of the thesis was to verify the effects of combined therapy on cell viability and apoptosis. It has been previously shown that drugs can have a synergistic effect on different type of cancer (Cao N, *et al*, 2015). However, the literature lacks information about the combination between HSPIs and Bortezomib on CML and AML cell lines; based on this and on the data previously

described, an attempt to understand the possible synergistic effect between these two families of drugs was performed. The approach chosen for the investigation on the combined therapy depended strongly from the results obtained when the cell lines were treated with the drugs as single agents. It was important to determine the concentrations of the two HSPIs and Bortezomib that needed to be combined on K562 and U937 cell lines. It seemed a sensible choice to choose 12.5 μM as a concentration for the two HSPIs, considering that it was the lowest dose to affect cell viability starting from one hour up to six hours on both cell lines and, potentially, the least cytotoxic. Bortezomib was generally more effective than the HSPIs as a single agent, and in the same fashion, the lowest but still effective doses were 15.6 nM and 31.2 nM. Therefore, the cells were treated for one hour with Pifithrin-μ or PES-CL and then the cells were treated with Bortezomib for 24 hours. Also, to compare the results and investigate which drug could enhance better the protein folding activity, Bortezomib was added to both cell lines and Pifithrin-μ and PES-CL were administered to the cells for 24 hours.

Following the experiments on cell viability, it appeared that the combination between Pifithrin-μ and Bortezomib is not synergistic. Indeed, when HSPIs are administered first, there is an effect on cell viability, particularly when Pifithrin-μ is added as single agent. This result appears to be equal for PES-CL first administration on K562 (Fig. 4.3.1.1 and Fig. 4.3.1.2.). These results may suggest that perhaps in an hour of treatment the HSPIs are able to already bind to their specific target HSP70 and affect the proteasome activity by accumulating near the proteasome, perhaps interfering with its activity and not allowing Bortezomib to bind to the proteasome. Although different in the choice of drugs, a study by Rodriguez, K. A., *et al* (2014) suggested that Pifithrin-μ seems to enhance the proteasome activity on naked mole rat cells by inhibiting HSP72 in combination with another proteasome inhibitor (MG-132), indicating that HSPIs and PIs could have a synergistic effect. The debate between synergy and antagonism between HSPIs and PIs on leukemic cell lines therefore still requires further knowledge.

The metabolism activity on U937 cells resulted more reduced than on K562; it was also clearer that HSPIs seem to not enhance Bortezomib activity. Both the HSPIs resulted to be more effective on cell viability when they were added to the cells as single agent; interestingly, the percentage of cells with affected cell viability where there was a combined treatment resulted the same as when the HSPIs were added as single agent (Fig. 4.3.2.1. and Fig. 4.3.3.1.). This clearly suggests that the HSPIs may bind to HSP70 and occupy the spaces on the proteasome where Bortezomib was supposed to bind to, therefore there was a competitive action between the two drugs. Although it is a different treatment, Pifithrin-α appeared to strongly inhibit pro-apoptotic proteins in combination with MAPKs inhibitors in human hepatoma PLC/PRF/5 (CD95-negative) cells; particularly Pifithrin-α and these inhibitors were pre-treated together to evaluate the effects of cinnamaldehyde, which induces caspase dependent apoptosis. Pifithrin-α in combination with MAPK inhibitors strongly affected pro-apoptosis protein levels; this suggests that Pifithrin-μ could be used in combination with MAPK inhibitors, which could be an interesting element of a future study (Wu, S. J., & Ng, L. T., 2007). However, Pifithrin-μ and Pifithrin-α both resulted to exert their activity if administered first, in support of the findings of this thesis. The antagonistic effect was perhaps even more evident when Bortezomib was added to the cells first; on one particular concentration, that is 12.5\15.6, the cell viability levels on both cell lines resulted higher than all the other concentrations added to the cells, single agent or the other combination (Fig. 4.3.2.2. and Fig. 4.3.3.2.). Again, in partial disagreement with these findings, Pifithrin-μ was demonstrated to affect cell viability in combination with TRAIL on

pancreatic cancer cell lines, indicating that it can have a synergistic effect with another drug (Monma H., *et al*., 2013). However, the drugs were added at the same time, not subsequently as it has been performed on this thesis. With this in mind, it is not possible to state if an eventual subsequent treatment similar to what suggested in this study could have had another result.

The analysis with the flow cytometer found partial confirms to what found on MTS assays; interestingly, on K562 apoptosis levels were higher when the drugs were combined, independently from the time of administration; it was particularly clear on PES-CL – Bortezomib experiments. Bortezomib single agent concentrations induced higher levels of apoptosis than combination concentrations (Fig. 4.3.5.1. and Fig. 4.3.5.3.). This was expected when Bortezomib was added first, not when PES-CL was the first drug to be added for one hour. This was not associated to what was previously found on cell viability assays; perhaps, the Annexin V\PI is a more specific and accurate assay than MTS assays and could give more detailed information on the state of the cells. However, on U937 cells Bortezomib showed an interfering activity; there was no significant difference between combination and Bortezomib single agents (Fig. 4.3.6.3.). This may suggest that Bortezomib concentrations can induce apoptosis independently from the administration of another drug on U937 following 24 hours subsequent treatment. Opposite to these findings, Bortezomib was demonstrated to act in a synergic fashion with histone deacetylase inhibitors; MM cells were treated with 6 hours of 2.4 nM Bortezomib before administration of the histone deacetylase inhibitors. This pretreatment significantly potentiates apoptosis on the cells (Pei X.Y., *et al*., 2004). The much lower dose of Bortezomib and the longer time of pre-treatment are to be considered as an important factor in this study. However, this thesis have demonstrate that one hour may be enough to induce apoptosis. When the HSPIs were added first, there was an improvement in the apoptosis levels when the drugs were combined, not confirming what found on MTS assays; indeed, considering what has been demonstrated on the previous chapter, Bortezomib is able to induce apoptosis to 66 % of U937 cells on both single agent treatment following only an hour treatment (Fig. 4.3.6.1. and Fig. 4.3.7.1.). The combined therapy did not improve these results, also considering a longer period of treatment. In disagreement with this finding, Yu C., *et al*., (2003) have demonstrated that Bortezomib (4.5 nM) and histone deacetylase inhibitors could induce more apoptosis in combination than when administered alone following 48 hours on K562 cells. Perhaps, this indicates that a longer time of treatment with Bortezomib may be beneficial in the induction of apoptosis and in the enhancement of HSPIs activity.

As suggested previously, these results further confirm that HSPIs and Bortezomib have an antagonistic effect and they do not enhance each other activity. When the HSPIs are added first, they perhaps bind to HSP72 and they accumulate HSP72 intracellularly near the proteasome, not allowing the bound with Bortezomib. When Bortezomib is added first, instead, perhaps it binds to the proteasome in one hour and the HSPIs with HSP70 cannot longer bind to the proteasome, therefore interfering with proteasome activity. This proposed mechanism is illustrated in Fig. 6.1.1. However, Pifithrin-μ and PES-CL mechanism of action and the involvement with HSP70 is not completely clear, it is hoped that this could be a possible explanation to a potential antagonistic effect between HSPIs and Bortezomib.

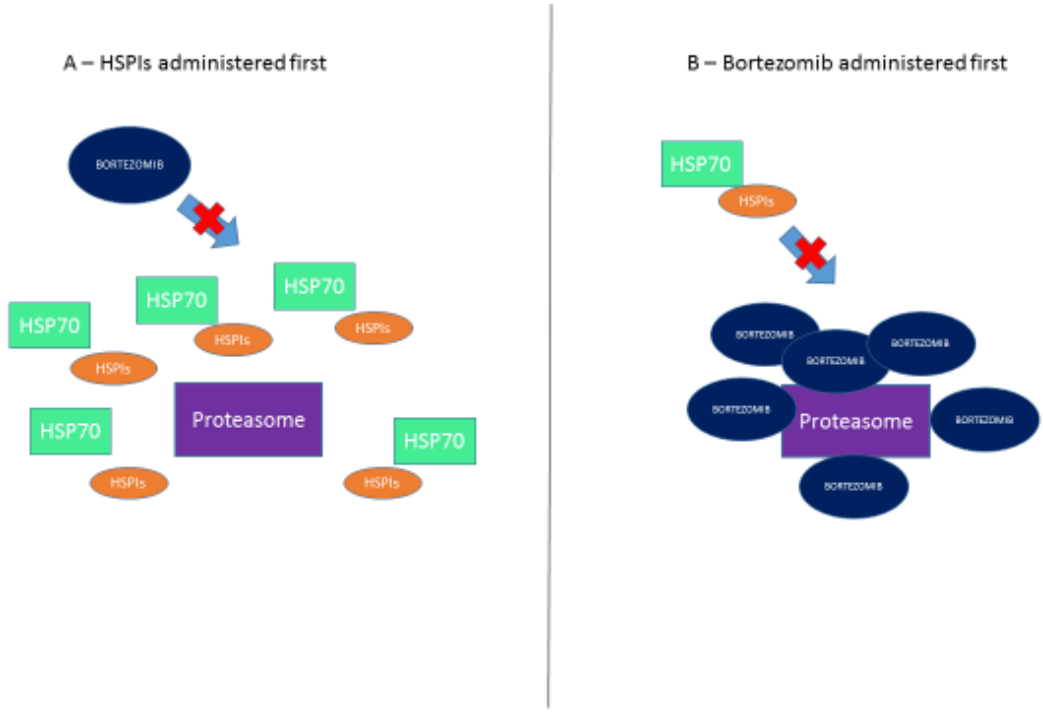


Fig.6.1.1. Mechanism of antagonistic effect between HSPIs and Bortezomib. (A)The administration of HSPIs as first drug causes the accumulation of HSP70 near the proteasome, not allowing the interaction between Bortezomib and the proteasome. (B) Bortezomib in one hour administration interacts with the proteasome, interfering with the activity of HSPIs and HSP70.

The third aim of the thesis was to investigate more the potential causes of the cell death, in this case, the HSP72 expression and its potential role in apoptosis. HSP72 is over-expressed on several types of cancer promoting cell survival (Goloudina A.R., *et al*., 2012). The inhibition of HSP70 can be considered a potential target for treatment of leukaemia, as suggested by (Kaiser M. *et al*, 2011). Importantly, Chapter 3 showed how these drugs could start to affect cell viability and have effects on apoptosis starting from an hour of administration up to six hours. Considering these results, it was decided to continue to investigate the effects of the combined therapy with the doses successfully tested on both leukemic cell lines; also, it was determined to treat the cells in a subsequent fashion for a maximum of six hours. With this in mind and considering the results described previously, HSP72 inhibition was evaluated following a combined treatment with HSPIs and Bortezomib. Particularly, HSP72 expression was analysed; it indeed belongs to HSP72 family of proteins and it is overexpressed following cellular stress or in cancer (Tavaria, M., *et al*., 1996). The HSPIs and Bortezomib appeared to specifically interact with HSP72, strongly inhibiting its activity. Generally, the interaction between HSPIs and Bortezomib to HSP72 appeared to inhibit the protein independently from the time of treatment and from the cell line.

Although not significant, there were small differences within the two cell lines. On K562, there was no notable difference in terms of HSP72 inhibition when Bortezomib or the HSPIs were added first; for example, the HSP72 inhibition on PES-CL-Bortezomib experiments was detected on almost all the cells treated, independently from which drug was added first. Importantly, there was no difference between the single agent concentrations and the combined concentrations, strongly suggesting and confirming that single agent treatment may be enough to treat leukemic cells. (Fig. 5.3.2.1. and Fig.

5.3.2.2.). It further confirmed what speculated in chapter 4, where the possible antagonistic effect of Pifithrin-μ and Bortezomib was demonstrated when Bortezomib is added first. Certainly, single agent treatments are able to selectively interact with HSP72 in a short time of treatment, suggesting a significant HSP72 inhibition in a time up to maximum 6 hours. This study partially confirmed what found by Leu J.I., *et al*., (2011), which suggested that Pifithrin-μ and Bortezomib have an antagonistic effect. When Pifithrin and Bortezomib were tested separately on different cell lines such as H1299 (human lung carcinoma cells) or A875 (human melanoma cells) for 24 hours to investigate 20 S proteasome activity, it was demonstrated that both drugs impair the proteasome function in the same fashion. Although the time of treatment was different, the results of this thesis tend to agree with what concluded by their group and the conclusion of antagonistic effect may found comfort in this study by Leu J.I., *et al*., (2011). This study could also be the first to indicate that a short time of treatment may be enough to inhibit HSP72 to significant percentage of K562 cells treated.

Similar results were obtained on U937 cells, suggesting that HSP72 can be strongly inhibited on AML cell lines and, potentially, on an AML patient. On U937 it was more evident, perhaps, the antagonism between the two drugs. Although still significant, when the drugs were combined there was a decrease in the HSP72 inhibition indicating a potential contrast between the two drugs activities. The proposed antagonism was particularly notable when Bortezomib was added first followed by 6 hours PES-CL treatment, where it reached the lowest percentage of inhibition, on both combined concentration (Fig. 5.3.4.2.). A study by Yerlikaya A., *et al*., (2010) suggested that HSP72 inhibitor quercetin could enhance the activity of proteasome inhibitor MG-132 on B16F10 melanoma cell line; this was not found for the HSPIs and the proteasome inhibitor which has been used in this thesis, although Bortezomib and the HSPIs used are different drugs. However, this may indicate that the synergistic effect between HSPIs and PIs may exist; this needs further studies on leukemic cell lines. Considering that Bortezomib specifically interacts with the proteasome causing the aggregation of misfolded protein that cannot longer enter the proteasome (Hideshima T., *et al*., 2011), it was hoped that HSP72 inhibition from HSPIs could lead to an enhanced activity of Bortezomib. According to these results, what expressed previously and shown on Fig.6.1.1. could be further confirmed. Furthermore, this is the first study to test PES-CL as single agent and in combination with Bortezomib with respect of HSP72 inhibition on leukemic cell lines.

Following these results, it was concluded that HSP72 may play a crucial role in protein degradation and in the consequent apoptosis cascade. For these reasons, it was important to further confirm the death by apoptosis by investigating which signalling cascade may be involved following the treatment with HSPIs and Bortezomib on a leukemic cell line. Bcl-2 protein is believed to inhibit apoptosis in cancer, resulting over-expressed and therefore not allowing pro-apoptotic proteins such as Bax and Bak to induce mitochondrial permeabilization and release of cytochrome c. The eventual expression of Bcl-2 following a treatment could indicate how the cells are dying, specifically which type of apoptosis pathway may be responsible of the cell death (Chipuk, J. E., and Green, D. R., 2008 and Yang J. *et al*, 1997). Considering that HSP72 is inhibited by the HSPIs and Bortezomib, as suggested by the results mentioned on the previous section, pro-survival proteins are correctly degraded, restoring apoptosis pathways. It remained to be understood if the pathway would have been intrinsic\mitochondrial or extrinsic, hence the interest in Bcl-2 expression. Significantly, U937 cells showed to be more responsive than K562 cells to the treatment, with respect of cell viability, apoptosis induction and HSP72 inhibition. For this reason, it seemed interesting to perform further analysis on a much more responsive cell line.

Bcl-2 expression was found high on U937 cells, independently from the combined treatment administered, whether it was HSPIs first or Bortezomib first. Indeed, the highest levels of Bcl-2 expression was found when PES-CL was administered first followed by Bortezomib treatment; there was almost no inhibition of the proteins (Fig. 5.3.6.1.). This suggests that PES-CL may strongly affect proteasome inhibitor activity by stopping the degradation of Bcl-2, perhaps accumulating HSP70 near the proteasome. However, when Bortezomib is added first, the levels of Bcl-2 are reduced, indicating a partial inhibition (Fig.5.3.6.2.). This is in line with some studies which demonstrated that Bortezomib can activate pro-apoptotic proteins when administered alone, (Fennell, D. A., *et al*., 2008) and on pancreatic, lung, prostate and breast cancer cell lines (Fahy, B. N., *et al*., 2005); this may suggest that this combination may be a potential choice of treatment in the future. A partial inhibition of Bcl-2 expression was found also when Pifithrin-μ and Bortezomib were added to U937 cells; in this case, Pifithrin-μ does not appear to be potent enough to inhibit Bcl-2 activity completely (Fig. 5.3.5.1. and Fig. 5.3.5.2.). Partially supporting this finding, Pifithrin-μ seems to inhibit Bcl-2 expression also on large B-cell lymphoma; interestingly those cells resulted to die by apoptosis when Bcl-2 was inhibited by Bcl-2 inhibitors and not when Pifithrin-μ was administered alone (Drakos E. *et al*, 2010). Multiple myeloma cells resulted to show inhibited Bcl-2 when its specific inhibitor was added, result not obtained when Bortezomib was added single agent to the cells. Combined treatment with Bortezomib and Bcl-2 inhibitor resulted in expression of cytochrome c, suggesting the triggering of the mitochondrial pathway of apoptosis (Pei XY, *et al*., 2003). Paralleling the results of this study with this literature, it appears clear that Bortezomib added first can inhibit Bcl-2 activity, although not completely. It can be suggested that one hour is not enough for Bortezomib to completely restore proteasome activity, balancing the levels of pro and anti-apoptosis proteins belonging to Bcl-2 family. This does not find confirm with HSP72 inhibition mentioned previously; perhaps, not all the cells are dead following a short time of treatment and a longer time of treatment as shown in Chapter 4 may improve apoptosis levels.

However, K562 and U937 resulted to die by apoptosis, as indicated in Chapter 3, following a short time of treatment; the high Bcl-2 expression, especially when PES-CL is administered first indicated that perhaps the cells may die of a mechanism independent from the mitochondrial pathway (Fleiss B. *et al*., 2015). Indeed, it could be possible that HSPIs inhibit HSP72 which then could not exert its ligase activity, causing the stabilization of p50 and RelA (not degraded by the proteasome). Also, Bortezomib appears to target NFκb pathway, perhaps by stabilizing the Iκκ complex which stops the nuclear translocation and activation of NFκb protein, leading to consequential DNA fragmentation and cell death. Caspase 8 and caspase 9 may be involved in triggering the IκB stabilization, as also suggested by Chaudhary, P. M., *et al*., (2000). It is not completely clear how the HSPIs and Bortezomib seems to strongly inhibit HSP72 and the proteasome but fail to have a synergistic action; therefore, the drug that is added first interfere with the drug subsequently added and it is not able to bind to the specific target, not being longer able to exert its mechanism of action as it also shown on Fig. 6.1.1. This is reflected in the failed cleavage of the pro-apoptotic protein Bcl-2, which was expressed on U937; it could be suggested that the inhibition of NFκB that leads to caspase 8 and 9 activation may be a possible explanation for the apoptosis that still occurred, according to the results obtained in Chapter 3 following a short time of administration and Chapter 4, following 24 hours combined treatment; Fig. 6.1.2. summarises this proposed mechanism of extrinsic apoptosis pathway. However, further studies are needed to investigate more in details how HSP72 inhibition could affect apoptosis on leukemic cell lines.

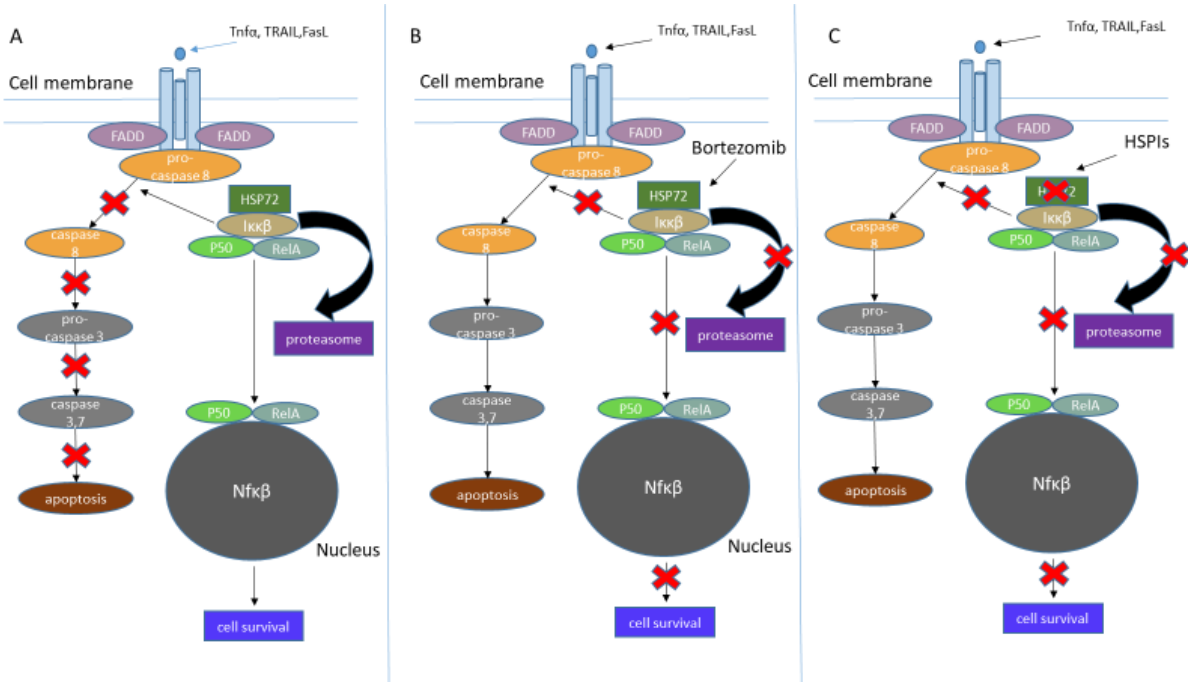


Fig. 6.1.2. Proposed mechanism of extrinsic apoptosis pathway following administration of HSPIs and Bortezomib on leukemic cells. A: Following stimuli from TNFα or TRAIL or FasL, the DISC complex is formed (FADD and pro-caspase 8). However, Ikkβ complex acts as a negative regulator of caspases and apoptosis cascade does not occur. At the same time, HSP72 transports Ikkβ to the proteasome, where it will be degraded. This event causes the translocation of p50 and RelA to the nucleus, inducing the over-expression of Nfκβ protein and consequent cell survival. **B**: Bortezomib selectively binds to the proteasome, stopping Ikkβ from degradation. Therefore, p50 and RelA are still bound to Ikkβ and caspases signalling cascade could lead to apoptosis. **C**: HSPIs inhibit HSP72 which, in return, is not able to transport Ikkβ to the proteasome for degradation. Pro-caspase 8 could then be cleavage by proteases into caspase 8 and the cells ultimately die by apoptosis.

6.2. Conclusion

Cell viability assays revealed that HSPIs like Pifithrin-μ and PES-CL affect the cell viability of K562 and U937 cells over a period of treatment of 24 hours. A further analysis demonstrated that one up to six hours may be sufficient to start killing leukemic cells; following three hours of treatment with Pifithrin-μ, the percentage of cell metabolic activity rose on both cell lines. Importantly one hour of Pifithrin-μ treatment is sufficient to start affecting cell viability on both cell lines. Indeed, throughout all the three concentrations there was an initial effect following this very short period on both leukemic cell lines. Generally, K562 cells generally seemed to be more resistant than U937. These are potentially the first findings that demonstrate that one hour of Pifithrin-μ administration is sufficient to affect cell viability on K562 and U937. Between the two HSPIs, PES-CL demonstrated to be more effective in respect of cell viability, especially on U937, either 24 hours or 6 hours treatment. Equally to what expressed for Pifithrin-μ, these are potentially the first findings to define a short time of administration and a low concentration for PES-CL as a single agent to two leukemic cell lines.

Apoptosis analysis confirmed the cell viability assays results; importantly, apoptosis was started following a short time of treatment and one hour appeared sufficient for the apoptosis to be triggered. Importantly, this was confirmed by statistical analysis on Pifithrin-μ treatment, which indicated that at the lowest concentration there is no statistical difference between an hour, three hours and six hours of treatment. Again, U937 showed to be generally more responsive to treatments with respect of apoptosis. These findings are potentially the first to identify the effects of one hour administration of Pifithrin-μ on apoptosis on leukemic cell lines.

Bortezomib as a single agent resulted significantly affect cell viability at low doses and following only one hour of treatment on both leukemic cell lines. Bortezomib therefore induces early apoptosis on both cell lines, following one hour of treatment. U937 cells is consistently more responsive to treatment compared to K562 cells, suggesting that treatment on K562 may need a more aggressive approach.

A combination of low doses of HSPIs and Bortezomib revealed an antagonist effect on both cell lines over a treatment of 24 hours. The drugs were alternatively administered to the cells for one hour and the other drug for the remaining 23 hours; it appeared that the cell viability was equally affected when the drugs were administered as single agent or as combination. This suggested that whichever drug was administered first, HSPIs or Bortezomib, protected the cells from the other drug activity, hence the antagonistic effect. The cell viability values were confirmed by Annexin V\PI assays; although some data could suggest a possible synergistic effect, especially on one particular combination, this was not confirmed by statistical analysis. This could potentially limit the understanding of the potential use of these two drugs in a combined therapy; although there was an improvement in apoptosis rate when the drugs were combined, the statistical data contradicted the positive outcome of the Annexin V results. Apoptosis assays also confirmed PES-CL superiority to Pifithrin-μ in effectiveness on both cell lines. Further, U937 appeared to be more sensible to treatment again.

HSP70 resulted strongly inhibited by HSPI's and Bortezomib on both cell lines; there was no significant difference between the treatments, either single or combined agents. This demonstrated

further the antagonism between the HSPIs and Bortezomib on both cell lines. It also reinforced the theory according to which any drug administered second is not able to enhance the activity of the drug first administered to the cells. This supported existing data found on other type of cancer. It could be concluded that 6 hours of treatment with low doses of HSPIs and Bortezomib are sufficient to inhibit HSP70 on K562 and U937. Also, the antagonistic effect or the not affected proteasome activity following combined therapy can be explained by suggesting that Pifithrin-μ could impair the proteasome activity by protein aggregation. In other words, Pifithrin-μ and Bortezomib could cause the aggregation of proteins when administered first and that could stop the other drug (Pifithrin-μ or Bortezomib) to interact with the proteasome and exert its mechanism of action. Furthermore, this is the first study to test PES-CL as single agent and in combination with Bortezomib with respect of HSP70 inhibition on leukemic cell lines.

High levels of Bcl-2 were found on U937 cells, particularly when PES-CL was administered first. Once again, there was no difference between the different concentrations, supporting the antagonistic results found previously. However, when Bortezomib was added first to both HSPIs, there was a reduction of Bcl-2 levels, indicating this as potential new treatment option. The expression of Bcl-2 and the apoptosis data of previous experiments suggested that U937 cells may die of extrinsic apoptosis, which did not involve the mitochondria and it is therefore independent from caspases activation. Therefore, there is the triggering of such extrinsic pathway of apoptosis, where Bcl-2 is not deprived of its pro-survival functions and yet K562 of U937 died by apoptosis. This could be in line with HSP70 inhibition which therefore cannot transport Bcl-2 to the proteasome, hence the failed cleavage of Bcl-2. Bortezomib, in particular, could accomplish so perhaps not directly inhibiting HSP70 but impeding the proteins transported by HSP70 to enter the proteasome by selectively inhibiting the 26S subunit of the proteasome. A novelty may be represented by a short time of treatment which could lead to an extrinsic pathway with low doses on U937 and, perhaps, on K562 CML cell line. With this in mind and considering the lack of literature in this respect, this study may give further knowledge about the combination of HSPIs and Bortezomib on U937 cells with respect to understanding the involvement of Bcl-2 in apoptosis. Further, it appears that this could also be the first study to further investigate PES-CL and apoptosis signalling cascade in depth. It could be concluded that single agent HSPIs and Bortezomib are not able to enhance their activity and that their single mechanism of action is able to induce apoptosis at low doses following a short time of treatment on leukemic cells. In summary, this thesis highlights and suggests the possibility of a novel therapy option with HSPIs and Bortezomib for patients with CML and AML. The scientific guidelines for treatment of CML and AML do not really consider these drugs; it is thus hoped that this work may represent a potential “stimuli” for expanding the treatment possibilities. Considering the percentage of patients who do not respond to current treatments, the possibility of having another option which is effective as it is shown here, could be comforting. However, further studies are needed to better clarify the mechanism of action of these drugs with respect of the apoptosis pathways on leukaemia cell lines.

6.3. Limitations

This thesis may possibly give further knowledge on the mechanisms of action of HSPIs and Bortezomib and, overall, offer a potential new therapy option to CML and AML patients. However, this study has some limitations. Firstly, different other CML and AML cell lines could also have been considered to further evaluate the drugs effects on apoptosis and cell viability; this thesis is limited to only targeting specific types of leukocytes (e.g. lymphocytes and monocytes). Also, there are missing informations on how long the cells need to reach exponential growth; this could have added more certainty to when administer drug to cells and therefore more value to the results of cell viability and apoptosis experiments.

The results described in this thesis represent a novelty and may offer potential new therapy solutions for CML and AML patients, as it is hoped it emerged in the previous chapters. However, not all the experiments performed during the research time have been described in this thesis. As for any other research and researcher, there have been some difficulties and technical issues. Perhaps the most important one is the not inclusion of western blots experiments; indeed, the western blot experiments aiming to veryify HSP72 expression were too uncertain or not presentable to be described on this work. Surely, these experiments could have been started earlier in the time course of the research, allowing more time to perform more experiments and finally include results on this study.

6.4. Future work

This thesis focused on the inhibition of HSP70 and its effects on apoptosis. It would be interesting to continue this study to find further information with respect of apoptosis signalling; levels of p53 protein could be evaluated to further confirm Bcl-2 expression on CML and AML cell lines. Also, pro-apoptotic proteins such as Bax and Bak could be analysed following the short time of treatment on CML and AML cell lines. The experiments could follow the exact same protocol that has been used for flow cytometry assays, as indicated in the Methods section of Chapter 5. Also, it could be interesting to detect p53 levels using a different technique such as Western Blot on the same cell lines.

Furthermore, given the results of this thesis, it would be interesting also to verify the commitment to apoptosis following a longer treatment than what used in this work. This would allow to understand if Bortezomib added first could restore proteasome activity as suggested in the final chapter. With this in mind, it would also be interesting to investigate proteasome activity through flow cytometry or western blot.

It could also be interesting to combine the HSPIs to other proteasome inhibitors, such as MG-132 which have been already tested on different cell lines. By doing that, a better comparison between Bortezomib mechanism of action and other proteasome inhibitors could be performed. A further experiment could also consist in the combined therapy with tyrosine kinase inhibitors and heat shock protein inhibitors; those inhibitors have been the first drug to be considered a targeted therapy drug. Throughout the years, their importance has grown in the therapy of myeloid leukaemia; it would be therefore interesting to test the same doses of HSPIs with tyrosine kinase inhibitors in vitro. Similarly it could be performed with Bortezomib too. It would also be interesting

to continue to investigate the effects of combination between HSPIs and Bortezomib on other type of leukaemia; this could potentially give other options for the treatment of ALL and CLL.

These treatments have been performed on leukemic cell lines; it would be therefore interesting to use these results as a model and perform the same experiments on patient samples. Particularly, it could be beneficial for a future potential study, to evaluate the combination of the drugs used in this work on human blood samples. Also, it could be interesting to obtain information on the difference in treatment response according to age and sex of the patients; this could potentially be incredibly useful for epidemiologic studies. It could also be interesting to test these drugs on samples of patients already resisting to treatment such as tyrosine kinase inhibitors, in order to potentially indicate a new treatment for patients in relapse.

If this research could be started again, or there could be more time and money, it would be interesting to evaluate the expression of the proteins belonging to the signalling cascade that ultimately allows p53 to be released. Indeed, probing the cells with proteins such as JAK-STAT, NfκB, or analyse PI3 levels could improve immensely the knowledge of the apoptosis signalling, downstream or upstream p53 release. Starting again today would probably present the same difficulties and issues that arose during the past years of the research; however, as in many other fields, experience could play an important role. In fact, knowing in advance the issues that could happen or knowing the techniques to use, would save time and money and allow to have better and more results.

Finally, the future perfect treatment for CML and AML is yet to be found. There is still not certainty on the causes of AML, in particular, and therefore finding a treatment that would improve life conditions and lead to a complete total remission for all patients is very challenging. However, there have been some improvements in the past decades; the dawning of the targeted therapy has immensely increased the chances of surviving CML and AML and improved life conditions, as a consequence. At the same time, CML and AML patients may develop resistance to what now has become ordinary targeted therapy. Researchers all over the world are currently hoping to understand how to overcome the relapse of patients and the mechanism of such resistance, to thus further targeting the reasons of not successful treatments. It is hoped that a work like this could help in the future doctors or pharmaceutical company to test these drugs on CML and AML patients at the concentrations showed on this thesis; ultimately, it could be potentially an alternative or maybe even more efficient treatment to finally defeat CML and AML and render these treatments accessible for all the patients who may need them.

CHAPTER 7: REFERENCES

A Baudino, T. (2015). Targeted cancer therapy: the next generation of cancer treatment. *Current drug discovery technologies*, *12*(1), 3-20.

Abe, M., Manola, J. B., Oh, W. K., Parslow, D. L., George, D. J., Austin, C. L., & Kantoff, P. W. (2004). Plasma levels of heat shock protein 70 in patients with prostate cancer: a potential biomarker for prostate cancer. *Clinical prostate cancer*, *3*(1), 49-53.

Adams, J. (2003). The proteasome: structure, function, and role in the cell. *Cancer treatment reviews*, *29*, 3-9.

Adams, J., Palombella, V. J., Sausville, E. A., Johnson, J., Destree, A., Lazarus, D. D., ... & Elliott, P. J. (1999). Proteasome inhibitors: a novel class of potent and effective antitumor agents. *Cancer research*, *59*(11), 2615-2622.

Aguayo, A., Kantarjian, H., Manshouri, T., Gidel, C., Estey, E., Thomas, D., ... & Freireich, E. (2000). Angiogenesis in acute and chronic leukemias and myelodysplastic syndromes. *Blood*, *96*(6), 2240-2245.

Andersson, L. C., Nilsson, K., & Gahmberg, C. G. (1979). K562—a human erythroleukemic cell line. *International journal of cancer*, *23*(2), 143-147.

Arora, A., & Scholar, E. M. (2005). Role of tyrosine kinase inhibitors in cancer therapy. *Journal of Pharmacology and Experimental Therapeutics*, *315*(3), 971-979.

Ashkenazi, A. (2008). Targeting the extrinsic apoptosis pathway in cancer. *Cytokine & growth factor reviews*, *19*(3-4), 325-331.

Attar, E.C., Johnson, J.L., Amrein, P.C., Lozanski, G., Wadleigh, M., DeAngelo, D.J., Kolitz, J.E., Powell, B.L., Voorhees, P., Wang, E.S. and Blum, W., (2012). Bortezomib added to daunorubicin and cytarabine during induction therapy and to intermediate-dose cytarabine for consolidation in patients with previously untreated acute myeloid leukemia age 60 to 75 years: CALGB (Alliance) study 10502. *Journal of clinical oncology*, *31*(7), 923-929.

Ávila-Arroyo, S., Nuñez, G. S., García-Fernández, L. F., & Galmarini, C. M. (2015). Synergistic effect of trabectedin and olaparib combination regimen in breast cancer cell lines. *Journal of breast cancer*, *18*(4), 329-338.

Balaburski, G. M., Julia, I., Leu, J., Beeharry, N., Hayik, S., Andrake, M. D., ... & Yen, T. (2013). A modified HSP70 inhibitor shows broad activity as an anticancer agent. *Molecular Cancer Research*, *11*(3), 219-229.

Banerji, U. (2009). Heat shock protein 90 as a drug target: some like it hot. *Clinical Cancer Research*, *15*(1), 9-14.

Bastian, L., Hof, J., Pfau, M., Fichtner, I., Eckert, C., Henze, G., ... & Shalapour, S. (2013). Synergistic activity of bortezomib and HDACi in preclinical models of B-cell precursor acute lymphoblastic leukemia via modulation of p53, PI3K/AKT, and NF-κB. *Clinical Cancer Research*, *19*(6), 1445-1457.

Ben-Neriah, Y. (2002). Regulatory functions of ubiquitination in the immune system. *Nature immunology*, *3*(1), 20.

Bhayat, F., Das-Gupta, E., Smith, C., & Hubbard, R. (2009). NSAID use and risk of leukaemia: a population-based case-control study. *Pharmacoepidemiology and drug safety*, *18*(9), 833-836.

Biswas, P., Delfanti, F., Bernasconi, S., Mengozzi, M., Cota, M., Polentarutti, N., Mantovani, A., Lazzarin, A., Sozzani, S. and Poli, G., (1998). Interleukin-6 induces monocyte chemotactic protein-1 in peripheral blood mononuclear cells and in the U937 cell line. *Blood*, *91*(1), 258-265.

Bitencourt, R., Zalcberg, I., & Louro, I. D. (2011). Imatinib resistance: a review of alternative inhibitors in chronic myeloid leukemia. *Revista brasileira de hematologia e hemoterapia*, *33*(6), 470-475.

Blagosklonny, M. V. (2002). Hsp-90-associated oncoproteins: multiple targets of geldanamycin and its analogs. *Leukemia*, *16*(4), 455.

Blagosklonny, M. V., Fojo, T., Bhalla, K. N., Kim, J. S., Trepel, J. B., Figg, W. D., ... & Neckers, L. M. (2001). The Hsp90 inhibitor geldanamycin selectively sensitizes Bcr-Abl-expressing leukemia cells to cytotoxic chemotherapy. *Leukemia*, *15*(10), 1537.

Blum, K. A., Johnson, J. L., Niedzwiecki, D., Canellos, G. P., Cheson, B. D., & Bartlett, N. L. (2007). Single agent bortezomib in the treatment of relapsed and refractory Hodgkin lymphoma: cancer and leukemia Group B protocol 50206. *Leukemia & lymphoma*, *48*(7), 1313-1319.

Bold, R. J., Virudachalam, S., & McConkey, D. J. (2001). Chemosensitization of pancreatic cancer by inhibition of the 26S proteasome. *Journal of Surgical Research*, *100*(1), 11-17.

Bostrom, B. (2016). Bortezomib for the treatment of acute lymphoblastic leukemia. *Expert Opinion on Orphan Drugs*, *4*(7), 775-780.

Breitschopf, K., Haendeler, J., Malchow, P., Zeiher, A. M., & Dimmeler, S. (2000). Posttranslational modification of Bcl-2 facilitates its proteasome-dependent degradation: molecular characterization of the involved signaling pathway. *Molecular and cellular biology*, *20*(5), 1886-1896.

Bucur, O., Stancu, A. L., Goganau, I., Petrescu, S. M., Pennarun, B., Bertomeu, T., ... & Khosravi-Far, R. (2013). Combination of bortezomib and mitotic inhibitors down-modulate Bcr-Abl and efficiently eliminates tyrosine-kinase inhibitor sensitive and resistant Bcr-Abl-positive leukemic cells. *PLoS One*, *8*(10), e77390.

Budina-Kolomets, A., Balaburski, G. M., Bondar, A., Beeharry, N., Yen, T., & Murphy, M. E. (2014). Comparison of the activity of three different HSP70 inhibitors on apoptosis, cell cycle arrest, autophagy inhibition, and HSP90 inhibition. *Cancer biology & therapy*, *15*(2), 194-199.

Budina-Kolomets, A., Webster, M. R., Leu, J. I.-J., Jennis, M., Krepler, C., Guerrini, A., … Murphy, M. E. (2016). HSP70 inhibition limits FAK-dependent invasion and enhances the response to melanoma treatment with BRAF inhibitors. *Cancer Research*, *76*(9), 2720–2730.

Burchert, A., Wang, Y., Cai, D., Von Bubnoff, N., Paschka, P., Müller-Brüsselbach, S., ... & Neubauer, A. (2005). Compensatory PI3-kinase/Akt/mTor activation regulates imatinib resistance development. *Leukemia*, *19*(10), 1774.

Cain, K., Bratton, S. B., & Cohen, G. M. (2002). The Apaf-1 apoptosome: a large caspase-activating complex. *Biochimie*, *84*(2), 203-214.

Calderwood, S. K., Xie, Y., Wang, X., Khaleque, M. A., Chou, S. D., Murshid, A., ... & Zhang, Y. (2010). Signal transduction pathways leading to heat shock transcription. *Signal transduction insights*, *2*, 13.

Cao, N., Cheng, D., Zou, S., Ai, H., Gao, J., & Shuai, X. (2011). The synergistic effect of hierarchical assemblies of siRNA and chemotherapeutic drugs co-delivered into hepatic cancer cells. *Biomaterials*, *32*(8), 2222-2232.

Carter, B.Z., Mak, P.Y., Wang, X., Yang, H., Garcia-Manero, G., Mak, D.H., Mu, H., Ruvolo, V.R., Qiu, Y., Coombes, K. and Zhang, N., (2017). Focal Adhesion Kinase as a Potential Target in AML and MDS. *Molecular Cancer Therapeutics*, *16*(6), 1133-1144.

Chalandon, Y., Thomas, X., Hayette, S., Cayuela, J. M., Abbal, C., Huguet, F., ... & Escoffre-Barbe, M. (2015). Randomized study of reduced-intensity chemotherapy combined with imatinib in adults with Ph-positive acute lymphoblastic leukemia. *Blood*, *125*(24), 3711-3719.

Chang, F., Lee, J. T., Navolanic, P. M., Steelman, L. S., Shelton, J. G., Blalock, W. L., ... & McCubrey, J. A. (2003). Involvement of PI3K/Akt pathway in cell cycle progression, apoptosis, and neoplastic transformation: a target for cancer chemotherapy. *Leukemia*, *17*(3), 590.

Chang, F., Steelman, L. S., Shelton, J. G., Lee, J. T., Navolanic, P. M., Blalock, W. L., ... & McCubrey, J. A. (2003). Regulation of cell cycle progression and apoptosis by the Ras/Raf/MEK/ERK pathway. *International journal of oncology*, *22*(3), 469-480.

Chaudhary, P. M., Eby, M. T., Jasmin, A., Kumar, A., Liu, L., & Hood, L. (2000). Activation of the NF-kB pathway by caspase 8 and its homologs. *Oncogene*, *19*(39), 4451-4460.

Chauhan, D., Catley, L., Li, G., Podar, K., Hideshima, T., Velankar, M., Mitsiades, C., Mitsiades, N., Yasui, H., Letai, A. and Ovaa, H. (2005). A novel orally active proteasome inhibitor induces apoptosis in multiple myeloma cells with mechanisms distinct from Bortezomib. *Cancer cell*, *8*(5), 407-419.

Chauhan, D., Singh, A., Brahmandam, M., Podar, K., Hideshima, T., Richardson, P., ... & Anderson, K. C. (2008). Combination of proteasome inhibitors bortezomib and NPI-0052 trigger in vivo synergistic cytotoxicity in multiple myeloma. *Blood*, *111*(3), 1654-1664.

Chen, D., Frezza, M., Schmitt, S., Kanwar, J., & P Dou, Q. (2011). Bortezomib as the first proteasome inhibitor anticancer drug: current status and future perspectives. *Current cancer drug targets*, *11*(3), 239-253.

Chen, D., Liu, X., Yang, Y., Yang, H., & Lu, P. (2015). Systematic synergy modeling: understanding drug synergy from a systems biology perspective. *BMC Systems Biology*, *9*, 56.

Chen, K. F., Chen, H. L., Tai, W. T., Feng, W. C., Hsu, C. H., Chen, P. J., & Cheng, A. L. (2011). Activation of phosphatidylinositol 3-kinase/Akt signaling pathway mediates acquired resistance to sorafenib in hepatocellular carcinoma cells. *Journal of Pharmacology and Experimental Therapeutics*, *337*(1), 155-161.

Chiorazzi, N., Rai, K. R., & Ferrarini, M. (2005). Chronic lymphocytic leukemia. *New England Journal of Medicine*, *352*(8), 804-815.

Chipoy, C., Brounais, B., Trichet, V., Battaglia, S., Berreur, M., Oliver, L., ... & Blanchard, F. (2007). Sensitization of osteosarcoma cells to apoptosis by oncostatin M depends on STAT5 and p53. *Oncogene*, *26*(46), 6653.

Chipuk, J. E., & Green, D. R. (2008). How do BCL-2 proteins induce mitochondrial outer membrane permeabilization?. *Trends in cell biology*, *18*(4), 157-164.

Colado, E., Álvarez-Fernández, S., Maiso, P., Martín-Sánchez, J., Vidriales, M.B., Garayoa, M., Ocio, E.M., Montero, J.C., Pandiella, A. and San Miguel, J.F., (2008). The effect of the proteasome inhibitor bortezomib on acute myeloid leukemia cells and drug resistance associated with the CD34+ immature phenotype. *Haematologica*, *93*(1), 57-66.

Colvin, G. A., & Elfenbein, G. J. (2003). The latest treatment advances for acute myelogenous leukemia. *Medicine and Health Rhode Island*, *86*(8), 243.

Coppo, P., Flamant, S., Mas, V. D., Jarrier, P., Guillier, M., Bonnet, M. L., ... & Turhan, A. G. (2006). BCR–ABL activates STAT3 via JAK and MEK pathways in human cells. *British journal of haematology*, *134*(2), 171-179.

Cortes, J. E., Talpaz, M., Giles, F., O'Brien, S., Rios, M. B., Shan, J., ... & Ferrajoli, A. (2003). Prognostic significance of cytogenetic clonal evolution in patients with chronic myelogenous leukemia on imatinib mesylate therapy. *Blood*, *101*(10), 3794-3800.

Coustan-Smith, E., Sancho, J., Hancock, M. L., Boyett, J. M., Behm, F. G., Raimondi, S. C., ... & Pui, C. H. (2000). Clinical importance of minimal residual disease in childhood acute lymphoblastic leukemia. *Blood*, *96*(8), 2691-2696.

Crawford, L. J., Walker, B., & Irvine, A. E. (2011). Proteasome inhibitors in cancer therapy. *Journal of cell communication and signaling*, *5*(2), 101-110.

Crawford, L. J., Windrum, P., Magill, L., Melo, J. V., McCallum, L., McMullin, M. F., ... & Irvine, A. E. (2009). Proteasome proteolytic profile is linked to Bcr-Abl expression. *Experimental hematology*, *37*(3), 357-366.

Creagh, E. M., Carmody, R. J., & Cotter, T. G. (2000). Heat shock protein 70 inhibits caspase-dependent and-independent apoptosis in Jurkat T cells. *Experimental cell research*, *257*(1), 58-66.

Csizmar, C. M., Kim, D. H., & Sachs, Z. (2016). The role of the proteasome in AML. *Blood cancer journal*, *6*(12), e503.

Daugaard, M., Rohde, M., & Jäättelä, M. (2007). The heat shock protein 70 family: Highly homologous proteins with overlapping and distinct functions. *FEBS letters*, *581*(19), 3702-3710.

de Labarthe, A., Rousselot, P., Huguet-Rigal, F., Delabesse, E., Witz, F., Maury, S., ... & Buzyn, A. (2007). Imatinib combined with induction or consolidation chemotherapy in patients with de novo Philadelphia chromosome–positive acute lymphoblastic leukemia: results of the GRAAPH-2003 study. *Blood*, *109*(4), 1408-1413.

de Oliveira, M. B., Fook-Alves, V. L., Eugenio, A. I., Fernando, R. C., Sanson, L. F. G., de Carvalho, M. F., ... & Colleoni, G. W. (2017). Anti-myeloma effects of ruxolitinib combined with bortezomib and lenalidomide: A rationale for JAK/STAT pathway inhibition in myeloma patients. *Cancer letters*, *403*, 206-215.

Deininger, M., Buchdunger, E., & Druker, B. J. (2005). The development of imatinib as a therapeutic agent for chronic myeloid leukemia. *Blood*, *105*(7), 2640-2653.

Dhillon, A. S., Hagan, S., Rath, O., & Kolch, W. (2007). MAP kinase signalling pathways in cancer. *Oncogene*, *26*(22), 3279-3290.

Drakos, E., Atsaves, V., Schlette, E., Li, J., Papanastasi, I., Rassidakis, G. Z., & Medeiros, L. J. (2009). The therapeutic potential of p53 reactivation by nutlin-3a in ALK+ anaplastic large cell lymphoma with wild-type or mutated p53. *Leukemia*, *23*(12), 2290-2299.

Druker, B. J., Guilhot, F., O'brien, S. G., Gathmann, I., Kantarjian, H., Gattermann, N., ... & Cervantes, F. (2006). Five-year follow-up of patients receiving imatinib for chronic myeloid leukemia. *New England Journal of Medicine*, *355*(23), 2408-2417.

Druker, B. J., Talpaz, M., Resta, D. J., Peng, B., Buchdunger, E., Ford, J. M., ... & Sawyers, C. L. (2001). Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia. *N Engl j Med*, *2001*(344), 1031-1037.

Duechler M, Linke A, Cebula B, Shehata M, Schwarzmeier JD, Robak T, Smolewski P. (2005) In vitro cytotoxic effect of proteasome inhibitor bortezomib in combination with purine nucleoside analogues on chronic lymphocytic leukaemia cells. *Eur J Haematol*.; 74(5):407-17.

Duncan, M. T., DeLuca, T. A., Kuo, H. Y., Yi, M., Mrksich, M., & Miller, W. M. (2016). SIRT1 is a critical regulator of K562 cell growth, survival, and differentiation. *Experimental cell research*, *344*(1), 40-52.

Dundas, S. R., Lawrie, L. C., Rooney, P. H., & Murray, G. I. (2005). Mortalin is over-expressed by colorectal adenocarcinomas and correlates with poor survival. *The Journal of pathology*, *205*(1), 74-81.

Duronio, V., Scheid, M. P., & Ettinger, S. (1998). Downstream signalling events regulated by phosphatidylinositol 3-kinase activity. *Cellular signalling*, *10*(4), 233-239.

Eipel, C., Schuett, H., Glawe, C., Bordel, R., Menger, M. D., & Vollmar, B. (2005). Pifithrin-alpha induced p53 inhibition does not affect liver regeneration after partial hepatectomy in mice. *Journal of hepatology*, *43*(5), 829-835.

Eiring, A. M., Page, B. D., Kraft, I. L., Mason, C. C., Vellore, N. A., Resetca, D., ... & Reynolds, K. R. (2015). Combined STAT3 and BCR-ABL1 inhibition induces synthetic lethality in therapy-resistant chronic myeloid leukemia. *Leukemia*, *29*(3), 586-597.

Elmore, S. (2007). Apoptosis: a review of programmed cell death. *Toxicologic pathology*, *35*(4), 495-516.

Esser, C., Alberti, S., & Höhfeld, J. (2004). Cooperation of molecular chaperones with the ubiquitin/proteasome system. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*, *1695*(1-3), 171-188.

Esser, C., Alberti, S., & Höhfeld, J. (2004). Cooperation of molecular chaperones with the ubiquitin/proteasome system. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*, *1695*(1), 171-188.

Faderl, S., Talpaz, M., Estrov, Z., O'Brien, S., Kurzrock, R., & Kantarjian, H. M. (1999). The biology of chronic myeloid leukemia. *New England Journal of Medicine*, *341*(3), 164-172.

Fahy, B. N., Schlieman, M. G., Mortenson, M. M., Virudachalam, S., & Bold, R. J. (2005). Targeting BCL-2 overexpression in various human malignancies through NF-κB inhibition by the proteasome inhibitor bortezomib. *Cancer chemotherapy and pharmacology*, *56*(1), 46-54.

Fang, J., Rhyasen, G., Bolanos, L., Rasch, C., Varney, M., Wunderlich, M., ... & Cortelezzi, A. (2012). Cytotoxic effects of bortezomib in myelodysplastic syndrome/acute myeloid leukemia depend on

autophagy-mediated lysosomal degradation of TRAF6 and repression of PSMA1. *Blood*, *120*(4), 858-867.

Fang, J., Rhyasen, G., Bolanos, L., Rasch, C., Varney, M., Wunderlich, M., ... & Cortelezzi, A. (2012). Cytotoxic effects of bortezomib in myelodysplastic syndrome/acute myeloid leukemia depend on autophagy-mediated lysosomal degradation of TRAF6 and repression of PSMA1. *Blood*, blood-2012.

Fennell, D. A., Chacko, A., & Mutti, L. (2008). BCL-2 family regulation by the 20S proteasome inhibitor bortezomib. *Oncogene*, *27*(9), 1189.

Fink, S. L., & Cookson, B. T. (2005). Apoptosis, pyroptosis, and necrosis: mechanistic description of dead and dying eukaryotic cells. *Infection and immunity*, *73*(4), 1907-1916.

Flaherty, K. T. (2006). Chemotherapy and targeted therapy combinations in advanced melanoma. *Clinical Cancer Research*, *12*(7), 2366s-2370s.

Fulda, S., & Debatin, K. M. (2006). Extrinsic versus intrinsic apoptosis pathways in anticancer chemotherapy. *Oncogene*, *25*(34), 4798.

Gabai, V. L., Budagova, K. R., & Sherman, M. Y. (2005). Increased expression of the major heat shock protein Hsp72 in human prostate carcinoma cells is dispensable for their viability but confers resistance to a variety of anticancer agents. *Oncogene*, *24*(20), 3328.

Gabai, V. L., Mabuchi, K., Mosser, D. D., & Sherman, M. Y. (2002). Hsp72 and stress kinase c-jun N-terminal kinase regulate the bid-dependent pathway in tumor necrosis factor-induced apoptosis. *Molecular and cellular biology*, *22*(10), 3415-3424.

Gampenrieder, S. P., Rinnerthaler, G., & Greil, R. (2013). Neoadjuvant chemotherapy and targeted therapy in breast cancer: past, present, and future. *Journal of oncology*, *2013*.

Gausdal, G., Gjertsen, B. T., Fladmark, K. E., Demol, H., Vandekerckhove, J., & Døskeland, S. O. (2004). Caspase-dependent, geldanamycin-enhanced cleavage of co-chaperone p23 in leukemic apoptosis. *Leukemia*, *18*(12), 1989-1996.

Goloudina, A. R., Demidov, O. N., & Garrido, C. (2012). Inhibition of HSP70: a challenging anti-cancer strategy. *Cancer letters*, *325*(2), 117-124.

Gomez-Bougie, P., Wuillème-Toumi, S., Ménoret, E., Trichet, V., Robillard, N., Philippe, M., Bataille, R. and Amiot, M., (2007). Noxa up-regulation and Mcl-1 cleavage are associated to apoptosis induction by bortezomib in multiple myeloma. *Cancer research*, *67*(11), 5418-5424.

Goss, V. L., Lee, K. A., Moritz, A., Nardone, J., Spek, E. J., MacNeill, J., ... & Polakiewicz, R. D. (2006). A common phosphotyrosine signature for the Bcr-Abl kinase. *Blood*, *107*(12), 4888-4897.

Granato, M., Lacconi, V., Peddis, M., Lotti, L. V., Renzo, L. D., Gonnella, R., … Cirone, M. (2013). HSP70 inhibition by 2-phenylethynesulfonamide induces lysosomal cathepsin D release and immunogenic cell death in primary effusion lymphoma. *Cell Death & Disease*, *4*(7), e730–.

Guillem V, Amat P, Collado M, Cervantes F, Alvarez-Larrán A, Martínez J, Tormo E, Eroles P, Solano C, Hernández-Boluda JC (2015). BCL2 gene polymorphisms and splicing variants in chronic myeloid leukemia. *Leukemia research*, *39*(11), 1278-1284.

Guo F, Sigua C, Bali P, George P, Fiskus W, Scuto A, Annavarapu S, Mouttaki A, Sondarva G, Wei S, Wu J. Mechanistic role of heat shock protein 70 in Bcr-Abl–mediated resistance to apoptosis in human acute leukemia cells. *Blood*, *105*(3), 1246-1255

Guo, F., Rocha, K., Bali, P., Pranpat, M., Fiskus, W., Boyapalle, S., ... & Lawrence, N. (2005). Abrogation of heat shock protein 70 induction as a strategy to increase antileukemia activity of heat shock protein 90 inhibitor 17-allylamino-demethoxy geldanamycin. *Cancer research*, *65*(22), 10536-10544.

Guo, N., & Peng, Z. (2013). MG132, a proteasome inhibitor, induces apoptosis in tumor cells. *Asia-Pacific Journal of Clinical Oncology*, *9*(1), 6-11.

Gurbuxani, S., Schmitt, E., Cande, C., Parcellier, A., Hammann, A., Daugas, E., Kouranti, I., Spahr, C., Pance, A., Kroemer, G. and Garrido, C., (2003). Heat shock protein 70 binding inhibits the nuclear import of apoptosis-inducing factor. *Oncogene*, *22*(43), 6669-6678.

Håkansson, P., Lassen, C., Olofsson, T., Baldetorp, B., Karlsson, A., Gullberg, U., & Fioretos, T. (2004). Establishment and phenotypic characterization of human U937 cells with inducible P210 BCR/ABL expression reveals upregulation of CEACAM1 (CD66a). *Leukemia*, *18*(3), 538.

Hallek, M., Fischer, K., Fingerle-Rowson, G., Fink, A. M., Busch, R., Mayer, J., ... & Bergmann, M. (2010). Addition of rituximab to fludarabine and cyclophosphamide in patients with chronic lymphocytic leukaemia: a randomised, open-label, phase 3 trial. *The Lancet*, *376*(9747), 1164-1174.

Hamilton, A. L., Eder, J. P., Pavlick, A. C., Clark, J. W., Liebes, L., Garcia-Carbonero, R., ... & Kinchla, N. (2005). Proteasome inhibition with bortezomib (PS-341): a phase I study with pharmacodynamic end points using a day 1 and day 4 schedule in a 14-day cycle. *Journal of Clinical Oncology*, 23(25), 6107-6116.

Hanlon, K., & Copland, M. (2017). Chronic myeloid leukaemia. *Medicine*.

Harousseau, J. L., Attal, M., Avet-Loiseau, H., Marit, G., Caillot, D., Mohty, M., ... & Michallet, M. (2010). Bortezomib plus dexamethasone is superior to vincristine plus doxorubicin plus dexamethasone as induction treatment prior to autologous stem-cell transplantation in newly diagnosed multiple myeloma: results of the IFM 2005-01 phase III trial. *Journal of Clinical Oncology*, *28*(30), 4621-4629.

Heaney, N. B., Pellicano, F., Zhang, B., Crawford, L., Chu, S., Kazmi, S. M., ... & Holyoake, T. L. (2010). Bortezomib induces apoptosis in primitive chronic myeloid leukemia cells including LTC-IC and NOD/SCID repopulating cells. *Blood*, *115*(11), 2241-2250.

Heaney, N. B., Pellicano, F., Zhang, B., Crawford, L., Chu, S., Kazmi, S. M., ... & Holyoake, T. L. (2010). Bortezomib induces apoptosis in primitive chronic myeloid leukemia cells including LTC-IC and NOD/SCID repopulating cells. *Blood*, blood-2008.

Heaney, N.B., Pellicano, F., Zhang, B., Crawford, L., Chu, S., Kazmi, S.M., Allan, E.K., Jorgensen, H.G., Irvine, A.E., Bhatia, R. and Holyoake, T.L (2010). Bortezomib induces apoptosis in primitive chronic myeloid leukemia cells including LTC-IC and NOD/SCID repopulating cells. *Blood*, *115*(11), 2241-2250.

Hideshima, T., Ikeda, H., Chauhan, D., Okawa, Y., Raje, N., Podar, K., ... & Anderson, K. C. (2009). Bortezomib induces canonical nuclear factor-κB activation in multiple myeloma cells. *Blood*, *114*(5), 1046-1052.

Hideshima, T., Richardson, P. G., & Anderson, K. C. (2011). Mechanism of action of proteasome inhibitors and deacetylase inhibitors and the biological basis of synergy in multiple myeloma. *Molecular cancer therapeutics*, *10*(11), 2034-2042.

Hirai, H., Sootome, H., Nakatsuru, Y., Miyama, K., Taguchi, S., Tsujioka, K., ... & Kotani, H. (2010). MK-2206, an allosteric Akt inhibitor, enhances antitumor efficacy by standard chemotherapeutic agents or molecular targeted drugs in vitro and in vivo. Molecular cancer therapeutics, 9(7), 1956-1967.

Ho, M. S., Medcalf, R. L., Livesey, S. A., & Traianedes, K. (2015). The dynamics of adult haematopoiesis in the bone and bone marrow environment. *British journal of haematology*, *170*(4), 472-486.

Hochhaus, A., Larson, R. A., Guilhot, F., Radich, J. P., Branford, S., Hughes, T. P., ... & Ortmann, C. E. (2017). Long-term outcomes of imatinib treatment for chronic myeloid leukemia. *New England Journal of Medicine*, *376*(10), 917-927.

Hoelbl, A., Schuster, C., Kovacic, B., Zhu, B., Wickre, M., Hoelzl, M. A., ... & Hennighausen, L. (2010). Stat5 is indispensable for the maintenance of bcr/abl-positive leukaemia. *EMBO molecular medicine*, *2*(3), 98-110.

Hoesel, B., & Schmid, J. A. (2013). The complexity of NF-κB signaling in inflammation and cancer. *Molecular cancer*, *12*(1), 86.

Hong, D. S., Banerji, U., Tavana, B., George, G. C., Aaron, J., & Kurzrock, R. (2013). Targeting the molecular chaperone heat shock protein 90 (HSP90): lessons learned and future directions. *Cancer treatment reviews*, *39*(4), 375-387.

Horton, T. M., Gannavarapu, A., Blaney, S. M., D'Argenio, D. Z., Plon, S. E., & Berg, S. L. (2006). Bortezomib interactions with chemotherapy agents in acute leukemia in vitro. *Cancer chemotherapy and pharmacology*, *58*(1), 13-23.

Hsu, S. M., Yang, C. H., Shen, F. H., Chen, S. H., Lin, C. J., & Shieh, C. C. (2015). Proteasome inhibitor bortezomib suppresses nuclear factor-kappa B activation and ameliorates eye inflammation in experimental autoimmune uveitis. *Mediators of inflammation*, *2015*.

Hsu, S. M., Yang, C. H., Shen, F. H., Chen, S. H., Lin, C. J., & Shieh, C. C. (2015). Proteasome inhibitor bortezomib suppresses nuclear factor-kappa B activation and ameliorates eye inflammation in experimental autoimmune uveitis. *Mediators of inflammation*, *2015*.

Hubbard, S. R., & Miller, W. T. (2007). Receptor tyrosine kinases: mechanisms of activation and signaling. *Current opinion in cell biology*, *19*(2), 117-123.

Hunger, S. P., Lu, X., Devidas, M., Camitta, B. M., Gaynon, P. S., Winick, N. J., ... & Carroll, W. L. (2012). Improved survival for children and adolescents with acute lymphoblastic leukemia between 1990 and 2005: a report from the children's oncology group. *Journal of Clinical Oncology*, *30*(14), 1663-1669.

Huse, D. M., Von Mehren, M., Lenhart, G., Joensuu, H., Blanke, C., Feng, W., ... & Demetri, G. (2007). Cost effectiveness of imatinib mesylate in the treatment of advanced gastrointestinal stromal tumours. *Clinical drug investigation*, *27*(2), 85-93.

Huse, D. M., Von Mehren, M., Lenhart, G., Joensuu, H., Blanke, C., Feng, W., ... & Demetri, G. (2007). Cost effectiveness of imatinib mesylate in the treatment of advanced gastrointestinal stromal tumours. *Clinical drug investigation*, *27*(2), 85-93.

Inaba, H., Greaves, M., & Mullighan, C. G. (2013). Acute lymphoblastic leukaemia. *The Lancet*, *381*(9881), 1943-1955.

Ishaq, M., Ojha, R., Sharma, K., Sharma, G., Singh, S. K., & Majumdar, S. (2016). Functional inhibition of Hsp70 by Pifithrin-μ switches Gambogic acid induced caspase dependent cell death to caspase independent cell death in human bladder cancer cells. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*, *1863*(11), 2560-2573.

Jäättelä, M., Wissing, D., Kokholm, K., Kallunki, T., & Egeblad, M. (1998). Hsp70 exerts its anti-apoptotic function downstream of caspase-3-like proteases. *The EMBO journal*, *17*(21), 6124-6134.

Jackson, N., Menon, B. S., Zarina, W., Zawawi, N., & Naing, N. N. (1999). Why is acute leukemia more common in males? A possible sex-determined risk linked to the ABO blood group genes. *Annals of hematology*, *78*(5), 233-236.

Jagannath, S., Durie, B. G., Wolf, J., Camacho, E., Irwin, D., Lutzky, J., ... & Crowley, J. (2005). Bortezomib therapy alone and in combination with dexamethasone for previously untreated symptomatic multiple myeloma. *British journal of haematology*, *129*(6), 776-783.

Jagannathan-Bogdan, M., & Zon, L. I. (2013). Hematopoiesis. *Development*, *140*(12), 2463-2467.

Jego, G., Hazoumé, A., Seigneuric, R., & Garrido, C. (2013). Targeting heat shock proteins in cancer. *Cancer letters*, *332*(2), 275-285.

Jego, G., Hazoumé, A., Seigneuric, R., & Garrido, C. (2013). Targeting heat shock proteins in cancer. *Cancer letters*, *332*(2), 275-285.

Jhaveri, K., Taldone, T., Modi, S., & Chiosis, G. (2012). Advances in the clinical development of heat shock protein 90 (Hsp90) inhibitors in cancers. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*, *1823*(3), 742-755.

Jung, J. Y., Kim, H. Y., Han, B., Choi, D. R., Zang, D. Y., & Kim, H. J. (2014). The positive effects of one-hour intravenous administration of Bortezomib on peripheral neuropathy in multiple myeloma patients. *BioMed research international*, *2014*.

Kaiser, M., Kühnl, A., Reins, J., Fischer, S., Ortiz-Tanchez, J., Schlee, C., ... & Thiel, E. (2011). Antileukemic activity of the HSP70 inhibitor pifithrin-μ in acute leukemia. *Blood cancer journal*, *1*(7), e28.

Kamal, A., Thao, L., Sensintaffar, J., Zhang, L., Boehm, M. F., Fritz, L. C., & Burrows, F. J. (2003). A high-affinity conformation of Hsp90 confers tumour selectivity on Hsp90 inhibitors. *Nature*, *425*(6956), 407-410.

Kampen, K. R. (2012). The discovery and early understanding of leukemia. *Leukemia research*, *36*(1), 6-13.

Kanno, S. I., Kurauchi, K., Tomizawa, A., Yomogida, S., & Ishikawa, M. (2015). Pifithrin-alpha has a p53-independent cytoprotective effect on docosahexaenoic acid-induced cytotoxicity in human hepatocellular carcinoma HepG2 cells. *Toxicology letters*, *232*(2), 393-402.

Kay, J., & High, W. A. (2008). Imatinib mesylate treatment of nephrogenic systemic fibrosis. *Arthritis & Rheumatology*, *58*(8), 2543-2548.

Kay, J., & High, W. A. (2008). Imatinib mesylate treatment of nephrogenic systemic fibrosis. *Arthritis & Rheumatism: Official Journal of the American College of Rheumatology*, *58*(8), 2543-2548.

Kayser, S., & Levis, M. J. (2014). FLT3 tyrosine kinase inhibitors in acute myeloid leukemia: clinical implications and limitations. *Leukemia & lymphoma*, *55*(2), 243-255.

Kiyoi, H., Naoe, T., Nakano, Y., Yokota, S., Minami, S., Miyawaki, S., ... & Akiyama, H. (1999). Prognostic implication of FLT3 and N-RAS gene mutations in acute myeloid leukemia. *Blood*, *93*(9), 3074-3080.

Kiyoi, H., Naoe, T., Nakano, Y., Yokota, S., Minami, S., Miyawaki, S., ... & Akiyama, H. (1999). Prognostic implication of FLT3 and N-RAS gene mutations in acute myeloid leukemia. *Blood*, *93*(9), 3074-3080.

Klikova, K., Pilchova, I., Stefanikova, A., Hatok, J., Dobrota, D., & Racay, P. (2016). The Role of Heat Shock Proteins in Leukemia. *Klin Onkol*, *29*, 29-38.

Kliková, K., Stefanikova, A., Pilchova, I., Hata, J., Chudy, P., Chudej, J., Dobrota, D. and Racay, P., (2015). Differential impact of bortezomib on HL-60 and K562 cells. *Gen Physiol Biophys*, *34*, 33-42.

Knight, T., & Irving, J. A. E. (2014). Ras/Raf/MEK/ERK pathway activation in childhood acute lymphoblastic leukemia and its therapeutic targeting. *Frontiers in oncology*, *4*.

Kono, H., & Rock, K. L. (2008). How dying cells alert the immune system to danger. *Nature Reviews Immunology*, *8*(4), 279-289.

Koretzky, G. A. (2007). The legacy of the Philadelphia chromosome. *Journal of Clinical Investigation*, *117*(8), 2030.

Kouroukis, T. C., Baldassarre, F. G., Haynes, A. E., Imrie, K., Reece, D. E., & Cheung, M. C. (2014). Bortezomib in multiple myeloma: systematic review and clinical considerations. *Current oncology*, *21*(4), e573.

Krakstad, C., & Chekenya, M. (2010). Survival signalling and apoptosis resistance in glioblastomas: opportunities for targeted therapeutics. *Molecular cancer*, *9*(1), 135.

Krukowski, K., Nijboer, C. H., Huo, X., Kavelaars, A., & Heijnen, C. J. (2015). Prevention of chemotherapy-induced peripheral neuropathy by the small-molecule inhibitor pifithrin-μ. *Pain*, *156*(11), 2184-2192.

Krukowski, K., Nijboer, C. H., Huo, X., Kavelaars, A., & Heijnen, C. J. (2015). Prevention of chemotherapy-induced peripheral neuropathy by the small-molecule inhibitor Pifithrin-μ. *Pain*, *156*(11), 2184–2192.

Langer, S. W. (2014). Dexrazoxane for the treatment of chemotherapy-related side effects. *Cancer management and research*, *6*, 357.

Lauricella, M., Emanuele, S., D'Anneo, A., Calvaruso, G., Vassallo, B., Carlisi, D., ... & Tesoriere, G. J. N. K. (2006). JNK and AP-1 mediate apoptosis induced by bortezomib in HepG2 cells via FasL/caspase-8 and mitochondria-dependent pathways. *Apoptosis*, *11*(4), 607-625.

Law, J. C., Ritke, M. K., Yalowich, J. C., Leder, G. H., & Ferrell, R. E. (1993). Mutational inactivation of the p53 gene in the human erythroid leukemic K562 cell line. *Leukemia research*, *17*(12), 1045-1050.

Lazaris, A. C., Theodoropoulos, G. E., Aroni, K., Saetta, A., & Davaris, P. S. (1995). Immunohistochemical expression of C-myc oncogene, heat shock protein 70 and HLA-DR molecules in malignant cutaneous melanoma. *Virchows Archiv*, *426*(5), 461-467.

Lee, D. M., Kim, I. Y., Seo, M. J., Kwon, M. R., & Choi, K. S. (2017). Nutlin-3 enhances the bortezomib sensitivity of p53-defective cancer cells by inducing paraptosis. *Experimental & Molecular Medicine*, *49*(8), e365–.

Leoni, F., Zaliani, A., Bertolini, G., Porro, G., Pagani, P., Pozzi, P., … Mascagni, P. (2002). The antitumor histone deacetylase inhibitor suberoylanilide hydroxamic acid exhibits antiinflammatory properties via suppression of cytokines. *Proceedings of the National Academy of Sciences of the United States of America*, *99*(5), 2995–3000.

Leu, J. J., Pimkina, J., Pandey, P., Murphy, M. E., & George, D. L. (2011). HSP70 inhibition by the small-molecule 2-phenylethynesulfonamide impairs protein clearance pathways in tumor cells. *Molecular Cancer Research*, *9*(7), 936-947.

Levis, M. (2013). FLT3 mutations in acute myeloid leukemia: what is the best approach in 2013?. *ASH Education Program Book*, *2013*(1), 220-226.

Levis, M. (2013). FLT3 mutations in acute myeloid leukemia: what is the best approach in 2013?. *ASH Education Program Book*, *2013*(1), 220-226.

Ling, X., Calinski, D., Chanan-Khan, A. A., Zhou, M., & Li, F. (2010). Cancer cell sensitivity to bortezomib is associated with survivin expression and p53 status but not cancer cell types. *Journal of Experimental & Clinical Cancer Research*, *29*(1), 8.

Llovet, J. M., & Hernandez-Gea, V. (2014). Hepatocellular carcinoma: reasons for phase III failure and novel perspectives on trial design. *Clinical cancer research*, *20*(8), 2072-2079.

Lum, M. A., Balaburski, G. M., Murphy, M. E., Black, A. R., & Black, J. D. (2013). Heat shock proteins regulate activation-induced proteasomal degradation of the mature phosphorylated form of protein kinase C. *Journal of Biological Chemistry*, *288*(38), 27112-27127.

Luo, J., Solimini, N. L., & Elledge, S. J. (2009). Principles of cancer therapy: oncogene and non-oncogene addiction. *Cell*, *136*(5), 823-837.

Luo, P., Lin, M., Li, L., Yang, B., & He, Q. (2011). The Proteasome Inhibitor Bortezomib Enhances ATRA-Induced Differentiation of Neuroblastoma Cells via the JNK Mitogen-Activated Protein Kinase Pathway. *PLoS ONE*, *6*(11), e27298.

Mahalka, A. K., Kirkegaard, T., Jukola, L. T., Jäättelä, M., & Kinnunen, P. K. (2014). Human heat shock protein 70 (Hsp70) as a peripheral membrane protein. *Biochimica et Biophysica Acta (BBA)-Biomembranes*, *1838*(5), 1344-1361.

Majid, A., Tsoulakis, O., Walewska, R., Gesk, S., Siebert, R., Kennedy, D. B. J., & Dyer, M. J. (2008). BCL2 expression in chronic lymphocytic leukemia: lack of association with the BCL2− 938A> C promoter single nucleotide polymorphism. *Blood*, *111*(2), 874-877.

Mañas, A., Wang, S., Nelson, A., Li, J., Zhao, Y., Zhang, H., Davis, A., Xie, B., Maltsev, N. and Xiang, J (2017). The functional domains for BaxΔ 2 aggregate-mediated caspase 8-dependent cell death. *Experimental cell research*, *359*(2), 342-355.

Marchenko, N. D., Wolff, S., Erster, S., Becker, K., & Moll, U. M. (2007). Monoubiquitylation promotes mitochondrial p53 translocation. *The EMBO journal*, *26*(4), 923-934.

Marley, S. B., Lewis, J. L., Schneider, H., Rudd, C. E., & Gordon, M. Y. (2004). Phosphatidylinositol-3 kinase inhibitors reproduce the selective antiproliferative effects of imatinib on chronic myeloid leukaemia progenitor cells. *British journal of haematology*, *125*(4), 500-511.

Mattiolo, P., Barbero-Farran, A., Amigó, J., Ripamonti, M., Ribas, J., & Boix, J. (2014). Cell death induced by 2-phenylethynesulfonamide uncovers a pro-survival function of BAX. *Cancer letters*, *354*(1), 115-121.

McKeon, A. M., Egan, A., Chandanshive, J., McMahon, H., & Griffith, D. M. (2016). Novel improved synthesis of HSP70 inhibitor, pifithrin-μ. In vitro synergy quantification of pifithrin-μ combined with Pt drugs in prostate and colorectal cancer cells. *Molecules*, *21*(7), 949.

Messinger, Y. H., Gaynon, P. S., Sposto, R., van der Giessen, J., Eckroth, E., Malvar, J., & Bostrom, B. C. (2012). Bortezomib with chemotherapy is highly active in advanced B-precursor acute lymphoblastic leukemia: Therapeutic Advances in Childhood Leukemia & Lymphoma (TACL) Study. *Blood*, *120*(2), 285-290.

Mohamed, M., Dun, K., & Grabek, J. (2013). Atypical features in a patient with acute promyelocytic leukaemia: a potential diagnostic pitfall. *BMJ case reports*, *2013*, bcr2013200152.

Mokhtari, R. B., Homayouni, T. S., Baluch, N., Morgatskaya, E., Kumar, S., Das, B., & Yeger, H. (2017). Combination therapy in combating cancer. *Oncotarget*, *8*(23), 38022–38043.

Monma H1, Harashima N, Inao T, Okano S, Tajima Y, Harada M. (2013) The HSP70 and autophagy inhibitor pifithrin-μ enhances the antitumor effects of TRAIL on human pancreatic cancer. *Mol Cancer Ther*. 12(4):341-51

Moreau, P., Pylypenko, H., Grosicki, S., Karamanesht, I., Leleu, X., Grishunina, M., ... & Arnulf, B. (2011). Subcutaneous versus intravenous administration of bortezomib in patients with relapsed multiple myeloma: a randomised, phase 3, non-inferiority study. *The lancet oncology*, *12*(5), 431-440.

Morey, T. M., Winick-Ng, W., Seah, C., & Rylett, R. J. (2017). Chaperone-mediated regulation of choline acetyltransferase protein stability and activity by HSC/HSP70, HSP90 and p97/VCP. *Frontiers in Molecular Neuroscience*, *10*, 415.

Mosser, D. D., Caron, A. W., Bourget, L., Denis-Larose, C., & Massie, B. (1997). Role of the human heat shock protein hsp70 in protection against stress-induced apoptosis. *Molecular and cellular biology*, *17*(9), 5317-5327.

Murphy, M. E. (2013). The HSP70 family and cancer. *Carcinogenesis*, *34*(6), 1181-1188.

Muszynski, K. W., Ruscetti, F. W., Heidecker, G., Rapp, U., Troppmair, J., Gooya, J. M., & Keller, J. R. (1995). Raf-1 protein is required for growth factor-induced proliferation of hematopoietic cells. *Journal of Experimental Medicine*, *181*(6), 2189-2199.

Nair, R. R., Tolentino, J. H., & Hazlehurst, L. A. (2012). Role of STAT3 in transformation and drug resistance in CML. *Frontiers in oncology*, *2*.

Nelson, E. A., Walker, S. R., Weisberg, E., Bar-Natan, M., Barrett, R., Gashin, L. B., ... & Ebert, B. L. (2011). The STAT5 inhibitor pimozide decreases survival of chronic myelogenous leukemia cells resistant to kinase inhibitors. *Blood*, *117*(12), 3421-3429.

Neviani, P., Santhanam, R., Trotta, R., Notari, M., Blaser, B. W., Liu, S., ... & Roy, D. C. (2005). The tumor suppressor PP2A is functionally inactivated in blast crisis CML through the inhibitory activity of the BCR/ABL-regulated SET protein. *Cancer cell*, *8*(5), 355-368.

Ng, L. T., & Wu, S. J. (2011). Antiproliferative activity of Cinnamomum cassia constituents and effects of pifithrin-alpha on their apoptotic signaling pathways in Hep G2 cells. *Evidence-based complementary and alternative medicine*, *2011*.

Niewerth, D., Dingjan, I., Cloos, J., Jansen, G., & Kaspers, G. (2013). Proteasome inhibitors in acute leukemia. *Expert review of anticancer therapy*, *13*(3), 327-337.

Nimmanapalli, R., O'Bryan, E., & Bhalla, K. (2001). Geldanamycin and its analogue 17-allylamino-17-demethoxygeldanamycin lowers Bcr-Abl levels and induces apoptosis and differentiation of Bcr-Abl-positive human leukemic blasts. *Cancer research*, *61*(5), 1799-1804.

Nowak, M., Tardivel, S., Sayegrih, K., Robert, V., Abreu, S., Chaminade, P., Vicca, S., Grynberg, A. and Lacour, B., (2011). Impact of polyunsaturated fatty acids on oxidized low density lipoprotein-induced U937 cell apoptosis. *Journal of atherosclerosis and thrombosis*, *18*(6), 494-503.

Nylandsted, J., Gyrd-Hansen, M., Danielewicz, A., Fehrenbacher, N., Lademann, U., Høyer-Hansen, M., ... & Jäättelä, M. (2004). Heat shock protein 70 promotes cell survival by inhibiting lysosomal membrane permeabilization. *Journal of Experimental Medicine*, *200*(4), 425-435.

Nylandsted, J., Rohde, M., Brand, K., Bastholm, L., Elling, F., & Jäättelä, M. (2000). Selective depletion of heat shock protein 70 (Hsp70) activates a tumor-specific death program that is independent of caspases and bypasses Bcl-2. *Proceedings of the National Academy of Sciences*, *97*(14), 7871-7876.

Obeng, E. A., Carlson, L. M., Gutman, D. M., Harrington, W. J., Lee, K. P., & Boise, L. H. (2006). Proteasome inhibitors induce a terminal unfolded protein response in multiple myeloma cells. *Blood*, *107*(12), 4907-4916.

Ochel, H.-J., Eichhorn, K., & Gademann, G. (2001). Geldanamycin: the prototype of a class of antitumor drugs targeting the heat shock protein 90 family of molecular chaperones. *Cell Stress & Chaperones*, *6*(2), 105–112.

Ocio, E. M., Vilanova, D., Atadja, P., Maiso, P., Crusoe, E., Fernández-Lázaro, D., … San-Miguel, J. F. (2010). *In vitro* and *in vivo* rationale for the triple combination of panobinostat (LBH589) and dexamethasone with either bortezomib or lenalidomide in multiple myeloma. *Haematologica*, *95*(5), 794–803.

Onuoha, S. C., Coulstock, E. T., Grossmann, J. G., & Jackson, S. E. (2008). Structural studies on the co-chaperone Hop and its complexes with Hsp90. *Journal of molecular biology*, *379*(4), 732-744.

Oscier, D. G., Gardiner, A. C., Mould, S. J., Glide, S., Davis, Z. A., Ibbotson, R. E., ... & Orchard, J. A. (2002). Multivariate analysis of prognostic factors in CLL: clinical stage, IGVH gene mutational status, and loss or mutation of the p53 gene are independent prognostic factors. *Blood*, *100*(4), 1177-1184.

Pakakasama, S., Kajanachumpol, S., Kanjanapongkul, S., Sirachainan, N., Meekaewkunchorn, A., Ningsanond, V., & Hongeng, S. (2008). Simple multiplex RT-PCR for identifying common fusion

transcripts in childhood acute leukemia. *International journal of laboratory hematology*, *30*(4), 286-291.

Papandreou, C. N., Daliani, D. D., Nix, D., Yang, H., Madden, T., Wang, X., ... & Kim, J. (2004). Phase I trial of the proteasome inhibitor bortezomib in patients with advanced solid tumors with observations in androgen-independent prostate cancer. *Journal of Clinical Oncology*, 22(11), 2108-2121.

Patury, S., Miyata, Y., & Gestwicki, J. E. (2009). Pharmacological targeting of the Hsp70 chaperone. *Current topics in medicinal chemistry*, *9*(15), 1337-1351.

Paul, M. K., & Mukhopadhyay, A. K. (2004). Tyrosine kinase–role and significance in cancer. *International journal of medical sciences*, *1*(2), 101.

Pei, X. Y., Dai, Y., & Grant, S. (2003). The proteasome inhibitor bortezomib promotes mitochondrial injury and apoptosis induced by the small molecule Bcl-2 inhibitor HA14-1 in multiple myeloma cells. *Leukemia*, *17*(10), 2036-2045.

Pérez-Galán, P., Roué, G., Villamor, N., Montserrat, E., Campo, E., & Colomer, D. (2006). The proteasome inhibitor bortezomib induces apoptosis in mantle-cell lymphoma through generation of ROS and Noxa activation independent of p53 status. *Blood*, *107*(1), 257-264.

Perkins, N. D. (2007). Integrating cell-signalling pathways with NF-κB and IKK function. *Nature reviews Molecular cell biology*, *8*(1), 49-62.

Pocaly, M., Lagarde, V., Etienne, G., Ribeil, J. A., Claverol, S., Bonneu, M., ... & Dupouy, M. (2007). Overexpression of the heat-shock protein 70 is associated to imatinib resistance in chronic myeloid leukemia. *Leukemia*, *21*(1), 93-101.

Poon, I. K. H., Hulett, M. D., & Parish, C. R. (2010). Molecular mechanisms of late apoptotic/necrotic cell clearance. *Cell Death & Differentiation*, *17*(3), 381-397.

Poulaki, V., Mitsiades, C. S., Kotoula, V., Negri, J., McMillin, D., Miller, J. W., & Mitsiades, N. (2007). The proteasome inhibitor bortezomib induces apoptosis in human retinoblastoma cell lines in vitro. *Investigative ophthalmology & visual science*, *48*(10), 4706-4719.

Proskuryakov, S. Y., & Gabai, V. L. (2010). Mechanisms of tumor cell necrosis. *Current pharmaceutical design*, *16*(1), 56-68.

Pui, C. H., Mullighan, C. G., Evans, W. E., & Relling, M. V. (2012). Pediatric acute lymphoblastic leukemia: where are we going and how do we get there?. *Blood*, *120*(6), 1165-1174.

Qin, J. Z., Xin, H., Sitailo, L. A., Denning, M. F., & Nickoloff, B. J. (2006). Enhanced killing of melanoma cells by simultaneously targeting Mcl-1 and NOXA. *Cancer Research*, *66*(19), 9636-9645.

Quaranta, M. T., Olivetta, E., Sanchez, M., Spinello, I., Paolillo, R., Arenaccio, C., ... & Labbaye, C. (2015). miR-146a controls CXCR4 expression in a pathway that involves PLZF and can be used to inhibit HIV-1 infection of CD4+ T lymphocytes. *Virology*, *478*, 27-38.

Reikvam, H., Hatfield, K. J., Ersvær, E., Hovland, R., Skavland, J., Gjertsen, B. T., ... & Bruserud, Ø. (2012). Expression profile of heat shock proteins in acute myeloid leukaemia patients reveals a distinct signature strongly associated with FLT3 mutation status–consequences and potentials for pharmacological intervention. *British journal of haematology*, *156*(4), 468-480.

Richardson, P. G., Xie, W., Mitsiades, C., Chanan-Khan, A. A., Lonial, S., Hassoun, H., ... & Kesari, S. (2009). Single-agent bortezomib in previously untreated multiple myeloma: efficacy, characterization of peripheral neuropathy, and molecular correlations with response and neuropathy. *Journal of Clinical Oncology*, *27*(21), 3518-3525.

Roccaro, A. M., Vacca, A., & Ribatti, D. (2006). Bortezomib in the treatment of cancer. *Recent patents on anti-cancer drug discovery*, *1*(3), 397-403.

Rodriguez, K. A., Osmulski, P. A., Pierce, A., Weintraub, S. T., Gaczynska, M., & Buffenstein, R. (2014). A cytosolic protein factor from the naked mole-rat activates proteasomes of other species and protects these from inhibition. *Biochimica et Biophysica Acta*, *1842*(11), 2060–2072.

Russo, M., Spagnuolo, C., Volpe, S., Mupo, A., Tedesco, I., & Russo, G. L. (2010). Quercetin induced apoptosis in association with death receptors and fludarabine in cells isolated from chronic lymphocytic leukaemia patients. *British journal of cancer*, *103*(5), 642-648.

Saha, M. N., Jiang, H., & Chang, H. (2010). Molecular mechanisms of nutlin-induced apoptosis in multiple myeloma: Evidence for p53-transcription-dependent and -independent pathways. *Cancer Biology & Therapy*, *10*(6), 567–578.

Samanta, A. K., Chakraborty, S. N., Wang, Y., Schlette, E., Reddy, E. P., & Arlinghaus, R. B. (2010). Destabilization of Bcr-Abl/Jak2 network by a Jak2/Abl kinase inhibitor ON044580 overcomes drug resistance in blast crisis chronic myelogenous leukemia (CML). *Genes & cancer*, *1*(4), 346-359.

Samanta, A. K., Lin, H., Sun, T., Kantarjian, H., & Arlinghaus, R. B. (2006). Janus kinase 2: a critical target in chronic myelogenous leukemia. *Cancer research*, *66*(13), 6468-6472.

Sanford, D. S., Kantarjian, H., O'Brien, S., Jabbour, E., Cortes, J., & Ravandi, F. (2015). The role of ponatinib in Philadelphia chromosome-positive acute lymphoblastic leukemia. *Expert review of anticancer therapy*, *15*(4), 365-373.

Saraste, A., Pulkki, K., Kallajoki, M., Heikkila, P., Laine, P., Mattila, S., ... & Voipio-Pulkki, L. M. (1999). Cardiomyocyte apoptosis and progression of heart failure to transplantation. *European journal of clinical investigation*, *29*(5), 380-386.

Satou, Y., Nosaka, K., Koya, Y., Yasunaga, J. I., Toyokuni, S., & Matsuoka, M. (2004). Proteasome inhibitor, bortezomib, potently inhibits the growth of adult T-cell leukemia cells both in vivo and in vitro.

Saußele, S., & Silver, R. T. (2015). Management of chronic myeloid leukemia in blast crisis. *Annals of hematology*, *94*(2), 159-165.

Sawyers, C. L., Hochhaus, A., Feldman, E., Goldman, J. M., Miller, C. B., Ottmann, O. G., ... & Fischer, T. (2002). Imatinib induces hematologic and cytogenetic responses in patients with chronic myelogenous leukemia in myeloid blast crisis: results of a phase II study. *Blood*, *99*(10), 3530-3539.

Scaffidi, P., Misteli, T., & Bianchi, M. E. (2002). Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. *Nature*, *418*(6894), 191.

Schlecht, R., Scholz, S.R., Dahmen, H., Wegener, A., Sirrenberg, C., Musil, D., Bomke, J., Eggenweiler, H.M., Mayer, M.P. and Bukau, B. (2013). Functional analysis of Hsp70 inhibitors. *PloS one*, *8*(11), e78443.

Schmid, J. A., & Birbach, A. (2008). IκB kinase β (IKKβ/IKK2/IKBKB)—A key molecule in signaling to the transcription factor NF-κB. *Cytokine & growth factor reviews*, *19*(2), 157-165.

Schnittger, S., Schoch, C., Dugas, M., Kern, W., Staib, P., Wuchter, C., ... & Haferlach, T. (2002). Analysis of FLT3 length mutations in 1003 patients with acute myeloid leukemia: correlation to cytogenetics, FAB subtype, and prognosis in the AMLCG study and usefulness as a marker for the detection of minimal residual disease. *Blood*, *100*(1), 59-66.

Schrama, D., Reisfeld, R. A., & Becker, J. C. (2006). Antibody targeted drugs as cancer therapeutics. *Nature reviews Drug discovery*, *5*(2), 147-159.

Schuermann, J. P., Jiang, J., Cuellar, J., Llorca, O., Wang, L., Gimenez, L. E., ... & Hart, P. J. (2008). Structure of the Hsp110: Hsc70 nucleotide exchange machine. *Molecular cell*, *31*(2), 232-243.

Sekihara, K., Harashima, N., Tongu, M., Tamaki, Y., Uchida, N., Inomata, T., & Harada, M. (2013). Pifithrin-μ, an inhibitor of heat-shock protein 70, can increase the antitumor effects of hyperthermia against human prostate cancer cells. *PloS one*, *8*(11), e78772.

Selimovic, D., Porzig, B.B., El-Khattouti, A., Badura, H.E., Ahmad, M., Ghanjati, F., Santourlidis, S., Haikel, Y. and Hassan, M. (2013). Bortezomib/proteasome inhibitor triggers both apoptosis and autophagy-dependent pathways in melanoma cells. *Cellular signalling*, *25*(1), 308-318.

Seo, J. S., Park, Y. M., Kim, J. I., Shim, E. H., Kim, C. W., Jang, J. J., ... & Lee, W. H. (1996). T cell lymphoma in transgenic mice expressing the humanHsp70gene. *Biochemical and biophysical research communications*, *218*(2), 582-587.

Shah, N. P., Nicoll, J. M., Nagar, B., Gorre, M. E., Paquette, R. L., Kuriyan, J., & Sawyers, C. L. (2002). Multiple BCR-ABL kinase domain mutations confer polyclonal resistance to the tyrosine kinase inhibitor imatinib (STI571) in chronic phase and blast crisis chronic myeloid leukemia. *Cancer cell*, *2*(2), 117-125.

Shah, N. P., Nicoll, J. M., Nagar, B., Gorre, M. E., Paquette, R. L., Kuriyan, J., & Sawyers, C. L. (2002). Multiple BCR-ABL kinase domain mutations confer polyclonal resistance to the tyrosine kinase inhibitor imatinib (STI571) in chronic phase and blast crisis chronic myeloid leukemia. *Cancer cell*, *2*(2), 117-125.

Sledge Jr, G. W. (2005). What is targeted therapy?.

Somogyi, A. A., & Phillips, E. (2017). Genomic testing as a tool to optimise drug therapy. *Australian prescriber*, *40*(3), 101.

Stanulla, M., & Schrappe, M. (2009, January). Treatment of childhood acute lymphoblastic leukemia. In *Seminars in hematology* (Vol. 46, No. 1, pp. 52-63). WB Saunders.

Stewart, A. K., Rajkumar, S. V., Dimopoulos, M. A., Masszi, T., Špička, I., Oriol, A., ... & Goranova-Marinova, V. (2015). Carfilzomib, lenalidomide, and dexamethasone for relapsed multiple myeloma. *New England Journal of Medicine*, *372*(2), 142-152.

Stone, R. M., Mandrekar, S. J., Sanford, B. L., Laumann, K., Geyer, S., Bloomfield, C. D., ... & Lo-Coco, F. (2017). Midostaurin plus chemotherapy for acute myeloid leukemia with a FLT3 mutation. *New England Journal of Medicine*, *377*(5), 454-464.

Strom, E., Sathe, S., Komarov, P. G., Chernova, O. B., Pavlovska, I., Shyshynova, I., ... & Komarova, E. A. (2006). Small-molecule inhibitor of p53 binding to mitochondria protects mice from gamma radiation. *Nature chemical biology*, *2*(9), 474.

Sundström, C., & Nilsson, K. (1976). Establishment and characterization of a human histiocytic lymphoma cell line (U-937). *International journal of cancer*, *17*(5), 565-577.

Talpaz, M., Silver, R. T., Druker, B. J., Goldman, J. M., Gambacorti-Passerini, C., Guilhot, F., ... & Hochhaus, A. (2002). Imatinib induces durable hematologic and cytogenetic responses in patients with accelerated phase chronic myeloid leukemia: results of a phase 2 study. *Blood*, *99*(6), 1928-1937.

Tanaka, T., Shibazaki, A., Ono, R., & Kaisho, T. (2014). HSP70 mediates degradation of the p65 subunit of nuclear factor κB to inhibit inflammatory signaling. *Sci. Signal.*, *7*(356), ra119-ra119.

Taromi, S., Lewens, F., Arsenic, R., Sedding, D., Sänger, J., Kunze, A., Möbs, M., Benecke, J., Freitag, H., Christen, F. and Kaemmerer, D (2017). Proteasome inhibitor bortezomib enhances the effect of standard chemotherapy in small cell lung cancer. *Oncotarget*, *8*(57), 97061

Tavaria, M., Gabriele, T., Kola, I., & Anderson, R. L. (1996). A hitchhiker's guide to the human Hsp70 family. *Cell stress & chaperones*, *1*(1), 23.

Thompson, P. A., Tam, C. S., O'Brien, S. M., Wierda, W. G., Stingo, F., Plunkett, W., ... & Keating, M. J. (2016). Fludarabine, cyclophosphamide, and rituximab treatment achieves long-term disease-free survival in IGHV-mutated chronic lymphocytic leukemia. *Blood*, *127*(3), 303-309.

Tilahun, A. Y., Theuer, J. E., Patel, R., David, C. S., & Rajagopalan, G. (2010). Detrimental effect of the proteasome inhibitor, bortezomib in bacterial superantigen-and lipopolysaccharide-induced systemic inflammation. *Molecular Therapy*, *18*(6), 1143-1154.

Ungureanu, D., Saharinen, P., Junttila, I., Hilton, D. J., & Silvennoinen, O. (2002). Regulation of Jak2 through the ubiquitin-proteasome pathway involves phosphorylation of Jak2 on Y1007 and interaction with SOCS-1. *Molecular and cellular biology*, *22*(10), 3316-3326.

Vaseva, A. V., & Moll, U. M. (2009). The mitochondrial p53 pathway. *Biochimica et Biophysica Acta (BBA)-Bioenergetics*, *1787*(5), 414-420.

Vojtek, A. B., & Der, C. J. (1998). Increasing complexity of the Ras signaling pathway. *Journal of Biological Chemistry*, *273*(32), 19925-19928.

Volloch, V. Z., & Sherman, M. Y. (1999). Oncogenic potential of Hsp72. *Oncogene*, *18*(24), 3648.

Volloch, V. Z., & Sherman, M. Y. (1999). Oncogenic potential of Hsp72. *Oncogene*, *18*(24), 3648.

Voorhees, P. M., Dees, E. C., O'Neil, B., & Orlowski, R. Z. (2003). The proteasome as a target for cancer therapy. *Clinical cancer research*, *9*(17), 6316-6325.

Walton, M. I., Wilson, S. C., Hardcastle, I. R., Mirza, A. R., & Workman, P. (2005). An evaluation of the ability of pifithrin-α and-β to inhibit p53 function in two wild-type p53 human tumor cell lines. *Molecular cancer therapeutics*, *4*(9), 1369-1377.

Wang, C., & Youle, R. J. (2009). The role of mitochondria in apoptosis. *Annual review of genetics*, *43*, 95-118.

Wang, C., Gao, D., Guo, K., Kang, X., Jiang, K., Sun, C., ... & Sun, H. (2012). Novel synergistic antitumor effects of rapamycin with bortezomib on hepatocellular carcinoma cells and orthotopic tumor model. *BMC cancer*, *12*(1), 166.

Warsch, W., Kollmann, K., Eckelhart, E., Fajmann, S., Cerny-Reiterer, S., Hölbl, A., ... & Herrmann, H. (2011). High STAT5 levels mediate imatinib resistance and indicate disease progression in chronic myeloid leukemia. *Blood*, *117*(12), 3409-3420.

Warsch, W., Walz, C., & Sexl, V. (2013). JAK of all trades: JAK2-STAT5 as novel therapeutic targets in BCR-ABL1+ chronic myeloid leukemia. *Blood*, *122*(13), 2167-2175.

Whitman, S. P., Archer, K. J., Feng, L., Baldus, C., Becknell, B., Carlson, B. D., ... & Kolitz, J. E. (2001). Absence of the wild-type allele predicts poor prognosis in adult de novo acute myeloid leukemia with normal cytogenetics and the internal tandem duplication of FLT3. *Cancer research*, *61*(19), 7233-7239.

Whitman, S. P., Archer, K. J., Feng, L., Baldus, C., Becknell, B., Carlson, B. D., ... & Kolitz, J. E. (2001). Absence of the wild-type allele predicts poor prognosis in adult de novo acute myeloid leukemia with normal cytogenetics and the internal tandem duplication of FLT3: a cancer and leukemia group B study. *Cancer research*, *61*(19), 7233-7239.

Wiestner, A. (2015). The role of B-cell receptor inhibitors in the treatment of patients with chronic lymphocytic leukemia. *Haematologica*, *100*(12), 1495-1507.

Wu, S. J., & Ng, L. T. (2007). MAPK inhibitors and pifithrin-alpha block cinnamaldehyde-induced apoptosis in human PLC/PRF/5 cells. *Food and chemical toxicology*, *45*(12), 2446-2453.

Xie, S., Wang, Y., Liu, J., Sun, T., Wilson, M. B., Smithgall, T. E., & Arlinghaus, R. B. (2001). Involvement of Jak2 tyrosine phosphorylation in Bcr–Abl transformation. *Oncogene*, *20*(43), 6188.

Yamamoto, T., Ebisuya, M., Ashida, F., Okamoto, K., Yonehara, S., & Nishida, E. (2006). Continuous ERK activation downregulates antiproliferative genes throughout G1 phase to allow cell-cycle progression. *Current Biology*, *16*(12), 1171-1182.

Yang, J., Liu, X., Bhalla, K., Kim, C.N., Ibrado, A.M., Cai, J., Peng, T.I., Jones, D.P. and Wang, X., (1997). Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. *Science*, *275*(5303), 1129-1132.

Ye, D., Wolff, N., Li, L., Zhang, S., & Ilaria, R. L. (2006). STAT5 signaling is required for the efficient induction and maintenance of CML in mice. *Blood*, *107*(12), 4917-4925.

Yerlikaya, A., Okur, E., Şeker, S., & Erin, N. (2010). Combined effects of the proteasome inhibitor bortezomib and Hsp70 inhibitors on the B16F10 melanoma cell line. *Molecular medicine reports*, *3*(2), 333-339.

Yu, C., Rahmani, M., Conrad, D., Subler, M., Dent, P., & Grant, S. (2003). The proteasome inhibitor bortezomib interacts synergistically with histone deacetylase inhibitors to induce apoptosis in Bcr/Abl+ cells sensitive and resistant to STI571. *Blood*, *102*(10), 3765-3774.

APPENDIX A

MATERIALS

Table 1. List of equipment

EQUIPMENT	MANUFACTURER	CATALOGUE NUMBER
MSC-Advantage™ Class II Biological Safety Cabinet	Thermo Fisher Scientific (Runcorn, UK)	51025411
EVOS auto FL microscope	Thermo Fisher Scientific (Runcorn, UK)	AMAFD1000
TC120 heated circulating water bath	Grant Instruments (Royston, UK)	TC120-P5
EVOS XL core	Thermo Fisher Scientific (Runcorn, UK)	AMEX1000
EVOS auto FL onstage incubator	Thermo Fisher Scientific (Runcorn, UK)	AMC1000
Heraeus Multifuge x1	Thermo Fisher Scientific (Runcorn, UK)	75004210
Hermle Z323K refrigerated centrifuge	VWR International (Lutterworth, UK)	5210221
Sigma A 1-14 micro centrifuge	Sigma-Aldrich (Gillingham, UK)	12084
Vortex mixer mini	Thermo Fisher Scientific (Runcorn, UK)	GBI-900-010E
BD Accuri™ C6 flow cytometer	BD Biosciences (Warrington, UK)	
Bio-Tek Synergy™ HT Multi-Detection Microplate Reader	Labtech International Ltd. (Heathfield, UK)	SIAFR
Microtitre plate shaker, 120 VAC	Stuart (Staffordshire, UK)	

Table 2. List of consumables

CONSUMABLES	MANUFACTURER	CATALOGUE NUMBER
0.5 ml centrifuge tubes	Starlabs (Manchester, UK)	S1605-0000
1.5 ml centrifuge tubes	Starlabs (Manchester, UK)	S1615-5500
15 ml centrifuge tubes	SLS (Nottingham, UK)	SLS8102
50 mL centrifuge tubes	SLS (Nottingham, UK)	SLS8106
Tissue culture flask 25cm^3	Fisher Scientific (Loughborough, UK)	10568482
Tissue culture flask 75cm^3	Fisher Scientific (Loughborough, UK)	10364131
Microplate 96 well flat bottom, sterile with lid	Fisher Scientific (Loughborough, UK)	10687551
Microplate 96 wells V-bottomed, not sterile	Fisher Scientific (Loughborough, UK)	FB56424
Adhesive plate seal	Fisher Scientific (Loughborough, UK)	11524794
Coomassie (Bradford) Protein Assay Kit	Fisher Scientific (Loughborough, UK)	10270014

Table 3. List of reagent and equipment used for cell culture and cell counting

REAGENTS\EQUIPMENT	MANUFACTURER	CATALOGUE NUMBER
Antibiotic antimycotic solution (100x), stabilized	Sigma Aldrich (Gillingham, UK)	A5955-100ML
Fetal Bovine Serum	Fisher Scientific (Loughborough, UK)	11573397
RPMI 1640 with L-glutamine	Lonza (Slough, UK)	BE12-702F
Tryphan blue solution	Sigma-Aldrich (Gillingham, UK)	T8154-100ML
Bright-Line™ Haemocytometer	Sigma-Aldrich (Gillingham, UK)	Z359629
U937 cell line	ATCC (Teddington, UK)	CRL-1593.2
K562 cell line	Sigma-Aldrich (Gillingham, UK)	89121407

Table 4. List of drugs used for treatment in this study

DRUGS	MANUFACTURER	CATALOGUE NUMBER
Bortezomib	Selleck Chemicals (Munchen, Germany)	S1013-SEL
Pifithrin-μ	Sigma-Aldrich (Gillingham, UK)	P0122-5MG
PES-CL	Calbiochem (Nottingham, UK)	531067

Table 5. List of antibodies used in this study

ANTIBODIES	MANUFACTURER	CATALOGUE NUMBER
Annexin V FITC	BD Pharmingen (Warrington, UK)	556419
PI staining solution FITC	BD Pharmingen (Warrington, UK)	51-66211E
Anti-mouse\rat Bcl2 FITC	Invitrogen (Runcorn, UK)	11-6992-42
HSP70 antibody FITC	Stressmarq (Kennett, UK)	C92F3A-5

Table 6. List of reagents used for flow-cytometry assays

REAGENTS	MANUFACTURER	CATALOGUE NUMBER
10x Annexin V Binding Buffer	BD Pharmingen	51-66121E
DPBS Dulbecco's phosphate buffered saline without Ca^{2+} and MG^{2+}	Lonza (Slough, UK)	BE17-513f
BD Cytofix/Cytoperm™ fixation and permeablization solution	BD Biosciences (Warrington, UK)	51-2090KZ

Table 2.1.7. List of reagents used for MTS assays

REAGENTS	MANUFACTURER	CATALOGUE NUMBER
Phenazine Ethosulphate (PES)	Santa Cruz Biotechnologies (Wembley, UK)	SC-215699
CellTitre 96®Aqueous MTS Reagent Powder, 1g	Promega (Southampton, UK)	G1111